PCT

WORLD INTELLECTUAL PROPI
International Bi

WO 9608506A1

INTERNATIONAL APPLICATION PUBLISHED UNDER

(51) International Patent Classification 6 :
C07K 2/00, 4/00, 7/00

A1

(11) International Publication Number: WO 96/08506

(43) International Publication Date: 21 March 1996 (21.03.96)

(21) International Application Number: PCT/US95/11405

(22) International Filing Date: 18 September 1995 (18.09.95)

(30) Priority Data:
08/308,103 16 September 1994 (16.09.94) US

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: PHOSPHORUS-32 LABELING OF ANTIBODIES FOR CANCER THERAPY

(57) Abstract

Recombinant fusion proteins containing at least one domain which confers targeting properties on the fusion protein, together with at least one peptide sequence that is a substrate for a protein kinase, can be labeled with ^{32}P or ^{33}P by treatment with a protein kinase and a ^{32}P- or ^{33}P-labeled phosphate donor. The labeled targeting proteins bind specifically to diseased cells or tissue, which are killed by the radiation from the ^{32}P or ^{33}P.

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PHOSPHORUS-32 LABELING OF ANTIBODIES FOR CANCER THERAPY

Background of the Invention

The present invention relates to novel recombinant proteins which are useful for radiotherapy. In particular the invention relates to targeting proteins which contain peptide sequences that are substrates for protein kinase enzymes, and that are radiolabeled with ^{32}P or ^{33}P by treatment with a protein kinase and a ^{32}P- or ^{33}P-labeled phosphate donor. This invention also relates to methods of therapy using the labeled proteins.

Many radionuclides have been studied for their suitability for internal administration to patients in radiotherapy. Some radionuclide compounds, containing isotopes such as ^{131}I, can be given systemically, taking advantage of the fact that these elements tend to localize to particular tissues by virtue of their chemical properties. Other radionuclides, such as ^{198}Au and ^{103}Pd have been administered in a localized fashion, for instance to the site of a tumor. Most recent approaches, however, have focused on methods of delivering radionuclides to a preselected tissue by attaching the radionuclide to a targeting protein, usually an antibody, which will then localize to that tissue.

A large number of methods for linking radionuclides to antibodies have been developed. The chemical toxicity of many radionuclides means that complex methods must often be used to stably bind the isotope to an antibody. For example, to use ^{90}Y, which has many desirable radiochemical properties, a chelate must be synthesized and covalently bound to the antibody to stably link the radioisotope to the antibody.

One isotope which displays many of the same desirable features as ^{90}Y, but which has received little attention for targeted radiotherapy, is ^{32}P. ^{32}P is inexpensive, is readily available in high specific activity in a variety of labeled molecules, and has a therapeutically desirable half-life of 14 days. It is absorbed by the body and is

not readily excreted, and is therefore amenable to use in outpatient procedures. In addition, ^{32}P emits only β-radiation with an excellent depth penetration in tissue of approximately 6mm. Unlike many other radionuclides under consideration for targeted radiotherapy, it is not inherently toxic, and is currently used clinically in some non-targeted applications, for example for the treatment of ovarian cancer and polycythemia rubra vera.

Another radioisotope of phosphorus, ^{33}P, has received even less attention than ^{32}P. ^{33}P shares the same chemical properties as ^{32}P, and has similarly desirable radiochemical characteristics. It is available in high specific activity, and has a 25-day half life with a β-particle emission energy of 0.25 MeV, approximately 15% of the value of the β-emission energy of ^{32}P.

The reason radioactive phosphorus has received relatively little attention for targeted radiotherapy applications has been the difficulty of linking it to targeting proteins. Most of the methods currently known are non-specific and slow, and do not efficiently incorporate radionuclide into the targeting protein. Most importantly, the non-specificity of the methods means that the effect of labeling on the ability of the targeting protein to bind to its target is necessarily unpredictable.

One very general method of labeling proteins with ^{32}P is simply to incubate the protein with α-^{32}P-labeled nucleoside triphosphates. Schmidt *et al.*, *FEBS Lett.* 194:305 (1986). The mechanism for the labeling reaction is unknown. The method is slow and gives only poor incorporation of label (less than 1% of the protein molecules are labeled), and is thus too inefficient for therapeutic use.

A second general method of ^{32}P labeling is to incubate proteins with [γ-^{32}P]ATP or $H_3{}^{32}PO_4$ in the presence of chromium ions. Hwang *et al.*, *Biochim. Biophys. Acta* 882:331 (1986). This method is relatively rapid, but

gives an unknown level of label incorporation and also leaves toxic chromium ions bound to the proteins, which would be therapeutically unacceptable.

A third general method is the use of ^{32}P-diphenylphosphinothioic chloride as a reactive labeling compound. De Boer *et al.*, *Clin. Exp. Immunol.* 3:865 (1968). This reagent is thought to react with lysine residues in proteins to form a highly stable conjugate, but also associates non-covalently with the labeled protein. Although this method allows labeling of proteins to high specific activity, the labeling reagent is not commercially available, and its preparation requires a two-step synthesis with relatively large amounts of hazardous radioactive materials.

A less general method of ^{32}P labeling is the use of periodate-oxidized [α-^{32}P]ATP to affinity-label proteins containing an ATP-binding site. Clertant *et al.*, *J. Biol. Chem.* 257:6300 (1982). Because many targeting proteins which are of therapeutic interest, in particular antibodies, do not contain ATP-binding sites this method is therefore of little general utility.

A more recent method, intended for labeling antibodies for radiotherapy, involves the chemical conjugation of protein kinase substrate peptides to antibodies. Foxwell *et al.*, *Brit. J. Cancer* 57:489 (1988). The conjugates are labeled by treatment with [γ-^{32}P]ATP in the presence of the catalytic subunit of cAMP-dependent protein kinase (protein kinase A, PKA), which transfers ^{32}P-phosphate to a serine residue in the substrate peptide. Although in the published study the chemical cross-linking had no effect on the immunoreactivity of the antibody, this will not always be the case, and it likely that the cross-linking will often have a deleterious effect on the binding properties of an antibody or other targeting protein.

It is apparent therefore, that new methods for ^{32}P- and ^{33}P- labeling targeting proteins are greatly to be desired. In particular, new methods which are generally

applicable to a wide variety of targeting proteins and which do not compromise the binding abilities of these proteins are needed.

Summary of the Invention

It is therefore an object of this invention to provide recombinant proteins which are capable of binding specifically to a complementary molecular species, and which can readily be radiolabeled with ^{32}P or ^{33}P by treatment with a protein kinase and a ^{32}P- or ^{33}P-labeled phosphate donor.

It is a further object of this invention to provide purified DNA molecules encoding the proteins described above.

It is a further object of this invention to provide methods for producing recombinant proteins, which are capable of binding specifically to a complementary molecular species, and which can readily be radiolabeled with ^{32}P or ^{33}P by treatment with a protein kinase and a ^{32}P- or ^{33}P-labeled phosphate donor.

It is yet a further object of this invention to provide pharmaceutical compositions containing ^{32}P- or ^{33}P-labeled proteins for radiotherapy of patients suffering from a tumor or infectious lesion.

It is a still further object of this invention to provide kits for ^{32}P- or ^{33}P-labeling recombinant proteins that are capable of binding specifically to a complementary molecular species.

It is a still further object of this invention to provide a method of radiotherapy of a patient suffering from a tumor or an infectious lesion, wherein a protein that specifically binds to a complementary molecule or structure produced by, or associated with, a tumor or an infectious lesion, and radiolabeled with ^{32}P or ^{33}P, is parenterally injected into a human patient suffering from a tumor or infectious lesion.

In fulfilling the foregoing objects, there has been provided, in accordance with one aspect of the invention, a protein capable of binding specifically to a

complementary molecular species by virtue of a complementarity-determining region contained within the protein. The protein contains also a heterologous peptide sequence that is a substrate for a protein kinase, whereby the protein can be radiolabeled with ^{32}P or ^{33}P. In a preferred embodiment the proteini s a recombinant fusion protein. In another preferred embodiment the substrate peptide sequence is a substrate for at least one serine/threonine-specific or tyrosine-specific protein kinase. In yet another preferred embodiment the substrate peptide sequence is a substrate for at least one protein kinase chosen from the group consisting of: bovine heart protein kinase, protein kinase A, protein kinase C, calcium/calmodulin-dependent protein kinase, casein kinase II, phosphorylase kinase, EGF-receptor kinase, insulin receptor kinase, *src*, *abl*, *lck*, *fyn*, *yes*, and $p72^{syk}$. In still another preferred embodiment the substrate peptide sequence is selected from the group comprising Leu-Arg-Arg-Ala-Ser-Leu-Gly, Trp-Arg-Arg-Ala-Ser-Leu-Gly, Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val, Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu,
Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys,
Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly,
Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys, and Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu.

In accordance with another aspect of the invention these has been provided an isolated DNA molecule encoding a protein capable of binding specifically to a complementary molecular species by virtue of a complementarity-determining region contained within the protein. The protein contains also a heterologous peptide sequence that is a substrate for a protein kinase, whereby the said protein can be radiolabeled with ^{32}P or ^{33}P.

In accordance with yet another aspect of the invention there has been provided a method of producing a protein by cloning the isolated DNA molecule described

above. This method further comprises cloning the DNA molecule into an expression vector, transforming host cells with this expression vector, and recovering transformed host cells which express the protein described above. The method also comprises the step of culturing the transformed host cells and recovering the protein described above from the cultured host cells.

In accordance with still another aspect of the invention there has been provided a method of producing a ^{32}P- or ^{33}P-labelled protein comprising contacting a protein as described above with a ^{32}P- or ^{33}P-labelled phosphate or thiophosphate donor in the presence of a protein kinase which can phosphorylate the protein kinase substrate sequence in the protein. In a preferred embodiment the ^{32}P- or ^{33}P-labelled phosphate donor is selected from $\gamma^{32}P$-ATP, γthio-$\gamma^{32}P$-ATP, $\gamma^{33}P$-ATP, γthio-$\gamma^{33}P$-ATP.

In accordance with yet another aspect of the invention there is provided a kit for producing a ^{32}P- or ^{33}P- radiolabeled protein, comprising a protein as described above and a protein kinase which acts on a specific substrate peptide sequence in the protein.

In accordance with still another aspect of the invention there has been provided an improved method of radiotherapy of a patient suffering from a tumor or an infectious lesion, wherein a protein that specifically binds to a complementary molecule or structure produced by or associated with a tumor or an infectious lesion, and radiolabeled with ^{32}P or ^{33}P, is parenterally injected into a human patient suffering from such a tumor or infectious lesion.

In accordance with another aspect of the invention there has been provided a pharmaceutical composition, comprising an effective amount of a ^{32}P- or ^{33}P-labeled protein produced according to the methods described above, in a pharmaceutically acceptable sterile vehicle.

Brief Description of the Drawings

Figure 1 shows a schematic representation of the dicistronic gene encoding the F(ab)'/F(ab)'$_2$ fragment of humanized MN14.

Figure 2 shows the strategy for subcloning the VCHC hinge region into pSW1VHpoly.

Figure 3 shows the strategy for subcloning PelB into VKpBR and VK.CK into PelBpBR.

Figure 4 shows the strategy for combining the heavy and light chain-encoding genes to form MN14pBEV.

Figure 5 shows the DNA and amino acid sequences for the dicistronic gene encoding the Fab'/F(ab')$_2$ fragments of humanized MN14 antibody. The PelB and hinge regions are boxed in broken lines. CDR sequences are represented by "X"s and boxed in solid lines. Primers used for dideoxy sequencing are indicated by arrows.

Figure 6 shows the cloning strategy used to modify the hinge region of MN14pBEV to introduce the Kemptide-encoding sequence.

Figure 7 shows a schematic diagram of the structure of the hMN14 Fab-KPT plasmid.

Figure 8 shows HPLC analyses of IgG-derived hMN14Fab' and Fab'-kemptide fusion protein before and after labeling with ^{32}P. The proteins were analyzed on a BioSil SEC 250 column eluted with 0.2M sodium phosphate, 0.02% sodium azide at 1 ml/min monitored either at 280 nm (panels A,B,C) or by radiation detector (panels D,E): (A) shows hMN14 Fab' kemptide fusion protein; (B) shows IgG-derived hMN14Fab'; (C) shows IgG-derived hMN14 F(ab')$_2$; (D) shows [γ]^{32}P-ATP; (E) shows hMN14 Fab'-kemptide fusion protein after ^{32}P labeling with bovine heart protein kinase.

Figure 9 shows HPLC analyses before (A) and after (B) reacting with soluble CEA.

Figure 10 shows a comparative blocking assay for IgG-derived hMN14 Fab' (closed squares), hMN14 Fab'-kemptide fusion protein (open circles), and ^{32}P-labeled hMN14 Fab'-kemptide fusion protein (closed circles).

Detailed Description

The present invention provides a means of labeling targeting proteins with ^{32}P or ^{33}P without compromising the binding properties of the targeting protein. The labeled targeting proteins bind specifically to diseased cells or tissue, which are killed by the radiation from the ^{32}P or ^{33}P. The present invention provides recombinant fusion proteins that contain at least one domain which confers targeting properties on the fusion protein, together with at least one peptide sequence that is a substrate for a protein kinase. The domain which confers the targeting properties of the protein is referred to herein as a complementarity determining region. Treatment of the fusion protein with a protein kinase and a ^{32}P- or ^{33}P-labeled phosphate donor transfers ^{32}P- or ^{33}P-phosphate efficiently and specifically to predetermined amino acids in the kinase substrate sequence, thereby radiolabeling the targeting protein.

The invention also includes pharmaceutical compositions comprising an effective amount of at least one of the ^{32}P-or ^{33}P-labeled targeting proteins of the invention in combination with a pharmaceutically acceptable sterile vehicle, as described, for example, in Remingtons's Pharmaceutical Sciences; Drug Receptors and Receptor Theory, 18th ed., Mack Publishing Co., Easton, PA (1990).

For the purposes of chemical and enzymatic reactivity, ^{32}P and ^{33}P labeled molecules behave identically. It will be understood therefore that reference hereafter to labeling with ^{32}P will also encompass labeling with ^{33}P.

The targeting proteins of the invention preferentially bind to cells and tissues which are associated with a disease state and, by killing these cells or tissues, alleviate the disease state. This binding occurs to complementary molecules and structures

associated with or expressed on the surface of the diseased cells or tissue, which preferably are not associated with or expressed on the surface of healthy cells. More typically the complementary moieties will be present on healthy cells, but to a lesser extent than is observed in the disease state. For example, many myelomas show large increases in expression of the interleukin 6 (IL-6) receptor compared to normal tissue. ^{32}P-labeled proteins targeted at the IL-6 receptor will bind preferentially to myeloma cells, leading to a high effective concentration of ^{32}P and causing preferential cell killing at the site of the tumor. Another example is carcinoembryonic antigen (CEA) which is highly expressed on the surface of many tumors. A ^{32}P-labeled antibody or antibody fragment which binds to CEA will cause preferential cell killing at the tumor site.

A. Methods for Introducing a Protein Kinase Substrate into a Targeting Protein by Mutating the DNA Sequence Encoding the Protein.

(i) Cloning the gene encoding the targeting protein

The labeled fusion proteins of the current invention can be derived from any targeting protein which binds with specificity to molecules or tissue structures that are implicated in disease. Examples of such targeting proteins include, but are not limited to: monoclonal antibodies and antigen-binding fragments of monoclonal antibodies; lymphokines, cytokines, and peptide growth factors; and lymphokine or cytokine receptor antagonists. In a preferred embodiment the targeting protein is a recombinant antibody, and in another preferred embodiment is a humanized recombinant antibody fragment produced, for example, in *E. coli*.

The gene encoding the targeting protein must first be obtained. When the targeting protein contains a single polypeptide chain, such as in a lymphokine, cytokine, or peptide growth factor (hereinafter referred to as "growth factors" for convenience) and the amino

acid sequence of the binding protein is known, the gene can be obtained by relatively straightforward cloning techniques. See, for example, Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989). As an example, the gene encoding human basic fibroblast growth factor (bFGF) can be cloned from human pituitary mRNA by first-strand cDNA synthesis with random hexamer primers, followed by PCR amplification using suitable primers based on the 5' and 3' ends of the known bFGF DNA sequence. The PCR amplification products are cloned using standard techniques. See, for example, Ausubel *et al.* (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons (1987), ch.15.7. Synthetic genes encoding many growth factors are also commercially available, for instance from R&D Systems (Minneapolis, MN).

When the targeting protein is an antibody or antibody fragment, it is necessary to clone both the heavy and light chains of the antibody. Antibody molecules are composed of two identical copies of heavy chains and light chains, covalently interconnected by disulfide bonds. For a general discussion, see Schultz *et al.*, "Proteins II: Structure-Function Relationship of Protein Families," in TEXTBOOK OF BIOCHEMISTRY WITH CLINICAL CORRELATIONS, 3rd Ed., T.M. Devlin (ed.), Wiley & Sons, pp. 92-134 (1992); Turner *et al.*, "Antigen Receptor Molecules," in IMMUNOLOGY, 3rd Ed., Roitt *et al.* (eds.), Mosby, pp. 4.1-4.20 (1993).

In the most common type of antibody, IgG, the heavy chain has approximately 440 amino acids, while the light chain has about 220 amino acids. The amino acid sequences of the carboxyl-terminal one-half of the light chain and the carboxyl-terminal three-quarters of the heavy chain are highly conserved among antibodies with different antigen specificities. These conserved regions in the light and heavy chains are termed "constant regions" and are designated as CL and CH, respectively. The CH regions determine whether a particular antibody

belongs to the antibody class IgG, IgA, IgD, IgE, or IgM. The CH regions within a class of antibodies are homologous but differ significantly from the amino acid sequence of the CH regions of other antibody classes.

In contrast, the amino acid sequences of the amino-terminal one-half of the light chains and the amino-terminal one-quarter of the heavy chains are highly variable among antibodies with different antigen specificities. Particular regions within these variable segments are "hypervariable" and have been designated as complementarity determining regions because these regions form the antigen binding site that is complementary to the topology of the antigen structure.

Each heavy chain is associated with a light chain such that the amino-terminal ends of both chains are near each other and comprise an antigen binding site. Because there are two heavy chains and two light chains per antibody molecule, each molecule has two antigen binding sites, and is therefore bivalent.

Proteolytic cleavage can be used to fragment an antibody into small, functional units. For example, proteolytic cleavage of an IgG molecule with papain results in the cleavage of the antibody in the hinge peptide of each heavy chain. One product of papain digestion is the carboxyl-terminal one-half of the heavy chains which are bound covalently in a "crystallizable fragment" (Fc). The Fc fragment does not bind antigen. The other cleavage products consist of an amino-terminal segment of a heavy chain which is associated with an entire light chain. These amino-terminal, or "antigen binding fragments" (Fab) can bind antigen with an affinity similar to that of the intact antibody molecule. Several different antigen binding fragments can be prepared, such as: $F(ab')_2$, which retain a disulfide cross-link between the two parts of the antibody and are therefore still bivalent; the Fab and Fab' fragments, where single heavy and light chains are still covalently linked, but are univalent; and the Fv, where the heavy

and light chain normally are associated non-covalently, (although they can be engineered to be disulfide-linked), and the fragment is univalent.

Cloning of the entire antibody heavy and light chain sequences of a previously identified antibody can be achieved by known methods, using DNA or RNA from hybridoma cells which produce the antibody. For example, the rearranged genomic DNA encoding the antibody chains, including the naturally occurring promoter and regulatory sequences, can be cloned from the hybridoma. See Oi *et al.*, *Proc. Nat'l. Acad. Sci.* 80:825 (1984). Alternatively, standard cDNA cloning methods can be used. See Sikder *et al.*, *J. Immunol.* 135:4215 (1988).

A preferred method for cloning antibody genes is by the PCR. See Larrick *et al.*, "PCR Amplification of Antibody Genes" in NEW TECHNIQUES IN ANTIBODY GENERATION, *Methods 2(2)* (1991), p. 106, and Orlandi *et al.*, *Proc. Nat'l. Acad. Sci.* 86:3833 (1989). This method is particularly amenable to cloning antibody variable regions by using sets of degenerate oligonucleotide primers aimed at small, relatively conserved, segments of the antibody variable regions. Larrick *et al.*, *supra*. Since, as described above, fully functional antibody fragments can be prepared which contain only the variable regions of the heavy and light chain, cloning of the genes encoding these regions is sufficient to allow preparation of recombinant antibodies with full binding activity.

(ii) Selection of a protein kinase and mutation of the cloned gene to introduce a protein kinase substrate sequence.

Once the gene encoding the targeting protein has been obtained, it is mutated by addition of a DNA sequence encoding a peptide which is a substrate for a protein kinase. The first step in this process is selection of an appropriate kinase for carrying out the desired ^{32}P-

phosphate labeling step, followed by selection of an appropriate substrate sequence.

Protein kinases are defined as enzymes which phosphorylate the amino acid side chains of proteins. See, for example, Hanks, *et al.*, *Science* 241:42 (1988), and Hunter *et al.*, *Annu. Rev. Biochem.* 54:897 (1985). The two main families of protein kinases are serine/threonine kinases, which phosphorylate the hydroxyl group on the side chain of serine and/or threonine, and tyrosine kinases, which phosphorylate the phenolic hydroxyl group of tyrosine. These enzymes play crucial roles in cellular regulation, and many members of both kinase families have already been identified. See Hanks *et al.*, *supra*.

A protein kinase which is suitable for the practice of the current invention can be either a serine/threonine or tyrosine kinase. Preferably the kinase is either commercially available, or can be obtained by simple purification schemes from readily available starting materials, including *via* recombinant DNA technology. Examples of commercially available protein kinases are: Bovine heart protein kinase (Sigma, St. Louis, MO); Protein kinase A (Promega, Madison, WI; Boehringer Mannheim, Indianapolis, IN); Protein kinase A catalytic subunit (Promega; Boehringer Mannheim); Protein kinase C (Promega; Boehringer Mannheim; Calbiochem, La Jolla, CA); Protein kinase C α-isozyme (Life Technologies, Gaithersburg, MD); Casein kinase II (Promega); EGF receptor kinase (Promega); cGMP-dependent protein kinase (Promega); *v-src* (Oncogene Science, Uniondale, NY); Phosphorylase kinase (Life Technologies).

Examples of protein kinases which are readily purified from natural sources are: EGF-receptor kinase from the membranes of A431 cells (Erneaux *et al.*, *J. Biol. Chem.* 258:4137 (1983)); insulin receptor kinase from placental membranes (Kasuga *et al.*, *J. Biol. Chem.* 258:10973 (1983)), see also Cobb *et al.*, *J. Biol. Chem.* 264:18701 (1989); phosphorylase kinase from rabbit muscle

(Cohen, *Methods Enzymol.* 99:243 (1983)); Protein kinase A (Beabo *et al.*, *Methods Enzymol.* 38C:299 (1974)); PDGF receptor (Bishayee *et al.*, *Proc. Natl. Acad. Sci. USA* 83:6756 (1986)).

Examples or protein kinases which may be prepared by recombinant DNA methodology include: Protein kinase C-α (Goswami *et al.*, *Biotechniques* 10:626 (1991); $p56^{lck}$ (Eck *et al.*, *J. Cell Biochem. (1994)*; $p72^{syk}$ (Couture *et al.*, *Mol. Cell Biol.* 14:5249 (1994); MAP-kinase kinase (Mansour *et al.*, *Science* 265:966 (1994)); $p59^{fyn}$ (Panchamoorthy *et al.*, *Mol. Cell. Biol.* 14:6372 (1994); *yes* (Sukegawa *et al.*, *Mol. Cell. Biol.* 7:41 (1987); EGF receptor kinase (Wedegaertner *et al.*, *J. Biol. Chem.* 264:11346 (1989).

Members of the same protein kinase family differ in their substrate specificity, allowing the selective phosphorylation of appropriate amino acids within a protein. A great deal of research has been carried out to define this substrate specificity for many of the known kinases. Much of this work has measured the kinetic parameters of phosphorylation of synthetic peptides by kinases. See Kemp *et al.*, "Design and Use of Peptide Substrates for Protein Kinases" in METHODS IN ENZYMOLOGY, Vol. 200 (Academic Press, Orlando, Fla., 1991), p. 121. See also PEPTIDES AND PROTEIN PHOSPHORYLATION, B.E. Kemp, Ed. (Uniscience CRC Press, Boca Raton, Fla., 1990). The kinases used in these studies can be purified from natural sources or prepared by recombinant means. Excellent peptide substrates have been found for a large number of different protein kinases.

There are three general approaches used to identify a peptide substrate for a protein kinase. See Kemp *et al.*, (1991) *supra*. The most common approach begins with identification of the phosphorylation sites within known substrates of the kinase. These can include natural substrates of the enzyme, including autophosphorylation sites within the enzyme itself, or proteins which are

known to be good substrates for a large number of kinases, such as myelin basic protein or histones. Once the sequences of these sites are determined, peptide substrates can be designed and tested as substrates. A second approach is to use degenerate random peptide sequences as substrates and to isolate and sequence the most rapidly phosphorylated peptides. A third approach is to prepare substrates which are analogues of the "pseudosubstrate" regions of the kinase. Many kinases contain autoregulatory regions which bind to the active site of the enzyme and keep it catalytically inactive until some triggering mechanism causes dissociation of the regulatory region and activation of the kinase. It has been found that many of these kinase regulatory regions contain sequences which are exceedingly good kinase substrates once a single amino acid is changed to introduce a phosphorylatable amino acid, for example by substituting serine for alanine.

Assays to determine the suitability of a particular peptide as a substrate for a protein kinase can be carried out using standard techniques of enzymology. See Kemp *et al.*, (1991) *supra*, and references cited therein. Examples of peptide substrates for protein kinases include:

For Protein Kinase C: Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val, (House *et al.*, *Science* 238:1726 (1987)); Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu, (Yasuda *et al.*, *Biochem. Biophys. Res. Commun.* 166:1220 (1990)); Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys, (House *et al.*, *J. Biol. Chem.* 262:772 (1987)).

For EGF receptor kinase and *abl*: Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, (Casnellie *et al.*, *Proc. Nat'l. Acad. Sci. USA* 79:282 (1982)).

For calcium/calmodulin-dependent kinase II: Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys, (Pearson *et al.*, *J. Biol. Chem.* 260:14471 (1985)).

For casein kinase II: Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu, (Kuenzel *et al.*, *J. Biol. Chem.* 262:9136 (1987)).

For $p34^{cdc2}$: Glu-Gly-Val-Pro-Ser-Thr-Ala-Ile-Arg-Glu-Ile-Ser-Leu-Leu-Lys-Glu, (Norbury *et al.*, *Biochim. Biophys. Acta* 989:85 (1989)); Ala-Asp-Ala-Gln-His-Ala-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Lys-Asp-Phe, (Marshak *et al. J. Cell Biochem.* 45:391 (1991)).

Listings of peptide substrates for protein kinases are given in Kemp (1990) *supra*, which is hereby incorporated by reference in its entirety.

Once a suitable kinase and peptide substrate have been selected, the gene encoding the targeting protein must be mutated to include a segment encoding the kinase substrate sequence. This can be readily achieved by standard methods for site-directed mutagenesis. See Ausubel *et al.*, *supra*, ch. 8; Sambrook *et al.*, *supra*.

For example, when the targeting protein is a single chain protein such as a growth factor, the nucleotide sequence encoding the substrate peptide can be introduced by the PCR. Oligonucleotide primers are prepared which are complementary to the 5′ and 3′ ends of the targeting protein gene, but which also include the peptide-encoding sequence, either just prior to the 3′ stop codon, or just after the 5′ translation initiation codon. Unique restriction endonuclease sites can also be introduced into the primers to facilitate subsequent cloning steps. PCR amplification of the targeting protein gene will lead to PCR products which contain the peptide-encoding sequence, and which can be cloned by standard methods. See Ausubel *et al.*, *supra*, ch. 15.7.

Similarly, when the targeting protein is an antibody, a protein kinase substrate sequence can be introduced into the antibody heavy or light chain using an oligonucleotide containing the sequence encoding the desired peptide as a primer, and by using either DNA clones encoding the antibody, or RNA from cells that produce the antibody of interest, as a template. Also

see, Huse, "Combinatorial Antibody Expression Libraries in Filamentous Phage," in ANTIBODY ENGINEERING: A PRACTICAL GUIDE, C. Borrebaeck (ed.), W.H. Freeman and Company, pp. 103-120 (1992). Site-directed mutagenesis can be performed, for example, using the TRANSFORMER™ Site-Directed Mutagenesis Kit (Clontech; Palo Alto, CA) according to the manufacturer's instructions.

Alternatively, a kinase substrate sequence can be introduced into an immunoglobulin heavy or light chain by synthesizing a gene with mutually priming oligonucleotides in which one of the oligonucleotides contains the desired mutation. Techniques for the construction of large synthetic genes are well known to those in the art. See, for example, Uhlmann, *Gene 71*:29-40 (1988); Wosnick *et al.*, *Gene 60*:115-127 (1988); Ausubel *et al.*, *supra*.

In the case of recombinant antibodies it is preferred that the added peptide sequence be grafted onto the antibody downstream of the C-terminus of the antibody hinge region, as this region will be distant from the complementarity determining region and will have little effect on the binding properties of the antibody. In a preferred embodiment the recombinant antibody is a Fab' fragment, and in another preferred embodiment is a humanized Fab' fragment (see below).

When the targeting protein is a single chain protein it is preferred that the kinase substrate be grafted onto a part of the molecule which is not essential for its targeting ability. In many cases other mutated fusion proteins with full targeting ability will have been prepared from the targeting protein, and should be used for guidance as to whether to place the substrate sequence at the amino or carboxy terminus of the protein. For example, fully active IL-6 mutants have been prepared with large portions of the *Pseudomonas* exotoxin grafted onto the C-terminus. Siegall *et al.*, *J. Biol. Chem.* 265:16318 (1990). In other cases such guidance may not be available and both of the possible fusions may need to

be prepared to ensure that at least one of the mutated proteins retains the ability to bind to its cellular target.

The peptide substrate sequence need not be limited to a single copy in the fusion protein. Multiple repeats of the sequence can be engineered into the targeting protein by the methods described above, and will allow labeling of the fusion protein with a plurality of ^{32}P-labeled phosphates, thus increasing the specific activity (radioactivity/mol protein) of the protein. This will be advantageous for delivering the largest possible dose of radioactivity to a diseased cell or tissue.

In summary, a protein kinase substrate sequence can be introduced into any targeting protein if two requirements are met. First, the nucleotide sequence of the targeting protein of interest must be available in order to design a complementary oligonucleotide containing the desired mutation. Second, there must be access to either cloned antibody DNA or cells that produce the protein of interest.

B. Methods for Expressing and Isolating the Mutated Targeting Protein Product.

(i) Methods for expressing the protein

After mutating the nucleotide sequence, mutated DNA is inserted into a cloning vector for further analysis, such as confirmation of the DNA sequence. To express the polypeptide encoded by the mutated DNA sequence, the DNA sequence must be operably linked to regulatory sequences controlling transcriptional expression in an expression vector, and then introduced into either a prokaryotic or eukaryotic host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors include translational regulatory sequences, leader sequences which can direct nascent proteins into the endoplasmic reticulum for glycosylation and secretion, and a marker gene which is suitable for selection of cells that carry the expression vector.

Suitable promoters for expression in a prokaryotic host can be repressible, constitutive, or inducible. Suitable promoters are well-known to those of skill in the art and include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the *trp*, *recA*, heat shock, and *lacZ* promoters of *E. coli*, the α-amylase and the σ^{28}-specific promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the *int* promoter of bacteriophage lambda, the *bla* promoter of the ß-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters are reviewed by Glick, *J. Ind. Microbiol.* *1*:277-282 (1987); Watson *et al.*, MOLECULAR BIOLOGY OF THE GENE, 4th Ed., Benjamin Cummins (1987); Ausubel *et al.*, *supra*, and Sambrook *et al.*, *supra*.

A preferred prokaryotic host is *E. coli*. Preferred strains of *E. coli* include BMH71-18mutS, Y1088, Y1089, CSH18, ER1451, and ER1647 (see, for example, Brown (Ed.), MOLECULAR BIOLOGY LABFAX, Academic Press (1991)). An alternative preferred host is *Bacillus subtilus*, including such strains as BR151, YB886, MI119, MI120, and B170 (see, for example, Hardy, "Bacillus Cloning Methods," in DNA CLONING: A PRACTICAL APPROACH, Glover (Ed.), IRL Press (1985)).

Methods for expressing single chain proteins in bacteria, especially *E. coli*, are well known in the art. See, for example, Sambrook *et al.*, *supra*. In addition, many systems are commercially available which allow high level expression of recombinant proteins in *E. coli*, such as the T7 expression system (Novagen, Madison, WI or Promega, Madison, WI). Many commercial expression systems also allow expression of proteins fused in-frame with additional sequences which allow rapid affinity purification of the protein. Examples are the glutathione S-transferase (pGEX) fusion system (Pharmacia, Piscataway, NJ), the FLAG system (IBI, New Haven, CT), the maltose binding protein (MBP) system (New

England Biolabs, Beverley, MA), and the 6-His system (Qiagen, Chatsworth, CA). These "expression fusion" partners are typically linked to the protein of interest by an amino acid sequence which comprises a recognition motif for a site-specific protease, such as Factor X or enterokinase. Once the expression fusion protein is affinity purified it is treated with the protease to cleave off the unwanted expression fusion partner. In some cases the expression fusion protein is fully active and the cleavage is unnecessary.

Methods for producing antibody fragments in *E. coli* are also well-known to those of ordinary skill in the art. See, for example, Huse, "Combinatorial Antibody Expression Libraries in Filamentous Phage," in ANTIBODY ENGINEERING: A PRACTICAL GUIDE, C. Borrebaeck (Ed.), W.H. Freeman and Company, pp. 103-120 (1992); Ward, "Expression and Purification of Antibody Fragments Using *Escherichia coli* as a Host," *Id.* at pp. 121-138 (1992); Pack *et al*, *Bio/Technology* 11:1271 (1993). Those skilled in the art also know methods for producing in *E. coli* Fv fragments, which consist of variable regions of heavy and light chains. The Fv fragments can be held together non-covalently, or can be linked by a peptide linker or an engineered disulfide linkage. *Id.* Also, see Whitlow *et al.*, "Single-Chain Fv Proteins and their Fusion Proteins," in NEW TECHNIQUES IN ANTIBODY GENERATION, *Methods 2(2)* (1991). Moreover, expression systems for cloning antibodies in prokaryotic cells are commercially available. For example, the IMMUNO ZAP™ Cloning and Expression System (Stratagene Cloning Systems; La Jolla, CA) provides vectors for the expression of antibody light and heavy chains in *E. coli*.

In a preferred embodiment, recombinant antibodies of the current invention are produced in *E. coli* as Fab' fragments from a dicistronic gene comprised of the heavy chain Fd-encoding DNA fused to its hinge region and the light chain-encoding DNA. The discistronic gene is also preferably fused at its 5' terminus to a *pelB* signal

sequence, which directs secretion of expressed proteins into the periplasmic space of *E. coli*. Unlike the strongly reducing environment of the *E. coli* cytoplasm, the periplasmic space allows correct protein folding and disulfide bonding patterns of expressed proteins to occur. In addition, if antibody expression is allowed to continue to high levels, recombinant antibody "leaks" from the *E. coli* cell and can be purified directly from the culture medium.

The present invention preferably encompasses the expression of a mutated DNA sequence in prokaryotic cells, but it is also contemplated that the fusion proteins of the invention may be produced in eukaryotic cells, for example mammalian, insect, and yeast cells. Suitable mammalian host cells include COS-7 cells (ATCC CRL 1651), non-secreting myeloma cells (SP2/0-AG14; ATCC CRL 1581), Chinese hamster ovary cells (CHO-K1; ATCC CCL 61), rat pituitary cells (GH_1; ATCC CCL 82), HeLa S3 cells (ATCC CCL 2.2), and rat hepatoma cells (H-4-II-E; ATCC CRL 1548).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, and simian virus. In addition, promoters from mammalian expression products, such as immunoglobulins, actin, collagen, or myosin, can be employed. Alternatively, a prokaryotic promoter (such as the bacteriophage T3 RNA polymerase promoter) can be employed, wherein the prokaryotic promoter is regulated by a eukaryotic promoter (for example, *see* Zhou *et al.*, *Mol. Cell. Biol. 10*:4529-4537 (1990); Kaufman *et al.*, *Nucl. Acids Res. 19*:4485-4490 (1991)). Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the genes can be modulated.

In general, eukaryotic regulatory regions will include a promoter region sufficient to direct the initiation of RNA synthesis. Such eukaryotic promoters

include the promoter of the mouse metallothionein I gene (Hamer *et al.*, *J. Mol. Appl. Gen.* *1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, *Cell* *31*:355-365 (1982)); the SV40 early promoter (Benoist *et al.*, *Nature (London)* *290*:304-310 (1981)); the Rous sarcoma virus promoter (Gorman *et al.*, *supra*); the cytomegalovirus promoter (Foecking *et al.*, *Gene* *45*:101 (1980)); the yeast *gal4* gene promoter (Johnston, *et al.*, *Proc. Natl. Acad. Sci. (USA)* *79*:6971-6975 (1982); Silver, *et al.*, *Proc. Natl. Acad. Sci. (USA)* *81*:5951-5955 (1984)); and the IgG promoter (Orlandi *et al.*, *Proc. Natl. Acad. Sci. USA* *86*:3833-3837 (1989)).

Strong regulatory sequences are the most preferred regulatory sequences of the present invention. Examples of such preferred regulatory sequences include the SV40 promoter-enhancer (Gorman, "High Efficiency Gene Transfer into Mammalian cells," in DNA CLONING: A PRACTICAL APPROACH, Volume II, Glover (Ed.), IRL Press pp. 143-190 (1985)), the hCMV-MIE promoter-enhancer (Bebbington *et al.*, *Bio/Technology* *10*:169-175 (1992)), and antibody heavy chain promoter (Orlandi *et al.*, *Proc. Natl. Acad. Sci. USA* *86*:3833-3837 (1989)). Also preferred are the kappa chain enhancer for the expression of the light chain and the IgH enhancer (Gillies, "Design of Expression Vectors and Mammalian Cell Systems Suitable for Engineered Antibodies," in ANTIBODY ENGINEERING: A PRACTICAL GUIDE, C. Borrebaeck (Ed.), W.H. Freeman and Company, pp. 139-157 (1992); Orlandi *et al.*, *supra*).

The mutated antibody-encoding sequence and an operably linked promoter may be introduced into eukaryotic cells as a non-replicating DNA molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the protein may occur through the transient expression of the introduced sequence. Preferably, permanent expression occurs through the integration of the introduced sequence into the host chromosome.

For expression in a eukaryotic host it is preferred that the introduced sequence be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Several possible vector systems are available for this purpose. One class of vectors utilize DNA elements which provide autonomously replicating extra-chromosomal plasmids, derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, or SV40 virus. A second class of vectors relies upon the integration of the desired genomic or cDNA sequences into the host chromosome. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama, *Mol. Cell. Biol.* *3*:280 (1983), Sambrook *et al.*, *supra*, Ausubel *et al.*, *supra*, Bebbington *et al.*, *supra*, Orlandi *et al.*, *supra*, and Fouser *et al.*, *Bio/Technology* *10*:1121-1127 (1992); Gillies, *supra*. Genomic DNA expression vectors which include intron sequences are described by Orlandi *et al.*, *supra*. In addition, many expression systems for use in eukaryotic host cells are commercially available, for example from Invitrogen Corp. (San Diego, CA), Clontech (Palo Alto, CA) and Promega (Madison, WI).

When the targeting protein of the current invention is a protein such as a growth factor, it is preferred that the human protein sequence is used. When the targeting protein is an antibody or antibody fragment, it will often be derived from a rat or a mouse hybridoma, and therefore may be recognized as a foreign antigen by the human immune system, reducing the blood circulation half-life. In such cases it is preferred that the antibody be "humanized" for clinical use in humans, thereby obviating potential problems of immunogenicity associated with the non-human antibody and providing a longer blood circulation half-life. Humanized antibodies

are produced by transferring the complementarity determining regions of the heavy and light chains of the rat or mouse antibody into a human variable domain, and then substituting human residues in the framework regions of the rodent counterparts. Techniques for producing humanized antibodies are described for example in: Jones *et al.*, *Nature* 321:522 (1986); Riechmann *et al.*, *Nature* 332:323 (1988); Verhoeyen *et al.*, *Science* 239:1534 (1988); Carter *et al.*, *Proc. Nat'l Acad. Sci. USA* 89:4285 (1992); Sandhu *et al.*, *Crit. Rev. Biotech.* 12:437 (1992); Singer *et al.*, *J. Immunol.* 150:2844 (1993).

Alternatively, an antibody targeting protein of the present invention can be derived from human antibody fragments isolated from a combinatorial immunoglobulin library. See, for example, Barbas *et al.*, *METHODS: A Companion to Methods in Enzymology* 2:119 (1991), and Winter *et al.*, *Ann. Rev. Immunol.* 12:433 (1994). Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from Stratagene Cloning Systems (La Jolla, CA).

In addition, an antibody of the present invention can be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies upon antigenic challenge, and can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by: Green *et al.*, *Nature Genet.* 7:13 (1994); Lonberg *et al.*, *Nature* 368:856 (1994); Taylor *et al.*, *Int. Immun.* 6:579 (1994).

(ii) Methods for isolating the protein.

The methods used to isolate and purify the targeting protein-peptide substrate fusion protein will depend on the nature of the targeting protein and the type of expression system used.

For example, if a single chain (non-antibody) fusion protein is produced in *E. coli* using the *lacZ* promoter, the bacteria are grown in a suitable medium, e.g. LB, and the expression of the recombinant antigen induced by adding IPTG to the medium. After culturing the bacteria for a further period of between 2 and 24 hours, the cells are collected by centrifugation and washed to remove residual media. The bacterial cells are then lysed, for example, by disruption in a cell homogenizer and centrifuged to separate the dense inclusion bodies and cell membranes from the soluble cell components. This centrifugation can be performed under conditions whereby the dense inclusion bodies are selectively enriched by incorporation of sugars such as sucrose into the buffer and centrifugation at a selective speed. If the fusion protein is expressed in the inclusion bodies, as is the case in many instances, these can be washed in any of several solutions to remove some of the contaminating host proteins, then solubilized in solutions containing high concentrations of urea (eg 8M) or chaotropic agents such as guanidine hydrochloride in the presence of reducing agents such as ß-mercaptoethanol or DTT (dithiothreitol). At this stage it may be advantageous to incubate the fusion protein for several hours under conditions suitable for the protein to undergo a refolding process into the native conformation of the protein. Such conditions generally include low protein concentrations (less than 500 µg/ml), low levels of reducing agent, concentrations of urea less than 2 M and often the presence of reagents such as a mixture of reduced and oxidized glutathione which facilitate the interchange of disulphide bonds within the protein molecule. Following refolding, the fusion protein can then be purified further and separated from the refolding

mixture by chromatography on any of several supports including ion exchange resins, gel permeation resins or on a variety of affinity columns. If the fusion protein is in the soluble fraction of the cell lysate, it can be purified by standard means, including ammonium sulfate precipitation, and chromatography on various media as described above.

If the fusion protein is an antibody fragment produced from *E. coli* using the *pelB* leader sequence as described above, following induction of protein synthesis the recombinant antibody can be directly purified from the culture medium using an affinity resin such as the Quick MAb column manufactured by Sterogene (Hesperia, CA), following the manufacturer's instructions. If the fusion protein contains a domain which allows affinity purification, using one of the systems described above, purification is carried out on an affinity matrix following the manufacturer's instructions, followed by selective proteolytic cleavage of the affinity fusion partner. In a preferred embodiment the affinity fusion system is the 6His system (Qiagen, Chatsworth, CA) linked to the protein of interest by a sequence which is cleavable by the tobacco etch virus protease. After purification on a nickel-agarose matrix the recombinant protein is cleaved using recombinant tobacco etch virus protease which also carries a 6His motif (Life Technologies, Gaithersburg, MD). Passage of the reaction mixture over the nickel matrix again removes both the 6His domain by-product and the recombinant protease, leaving the desired protein essentially pure.

If the fusion protein is to be produced in a eukaryotic cell culture system, standard techniques of protein purification, such as those described above, can also be applied. See, for example, Burgess, "Protein Purification" in PROTEIN ENGINEERING, Oxender *et al.*, Eds. (Alan R. Liss, New York, 1987) pp.71-82 and references cited therein.

C. Methods for Phosphorylating the Targeting Protein.

Once the targeting protein containing the kinase substrate sequence has been purified, it must be phosphorylated using a protein kinase and a ^{32}P-labeled phosphate donor. Protocols for phosphorylation of proteins are well known in the art. Preferred reaction conditions have been developed for assay of the enzyme activity of most of the known kinases, and these can be adapted to suit the needs of the present invention. See PROTEIN PHOSPHORYLATION: A PRACTICAL APPROACH, Hardie, Ed. (IRL Press, Oxford, 1993).

When the kinase used is one that is commercially available, the phosphorylation is carried out according to the manufacturer's instructions. Normally it is desirable to allow the phosphorylation to proceed to stoichiometric completion, i.e. to phosphorylate every possible site in the fusion protein.

A preferred phosphate donor for all the kinases contemplated by the present invention is γ-^{32}P-ATP. It is also contemplated that the fusion proteins of the present invention can be thiophosphorylated using a nucleoside thiophosphate donor as described in WO 90/11289. Thiophosphorylated proteins are anticipated to be more resistant to the action of phosphatases *in vivo* than phosphates.

To separate the phosphorylated reaction product from the kinase and other reaction by-products standard chromatographic techniques such as size exclusion HPLC can be used. For ease of use, in a preferred embodiment the separation is carried out using a size-exclusion spin column, such as a Sephadex G-50-80 (Pharmacia, Piscataway, NJ).

To confirm that phosphorylation does not have an adverse effect on the binding properties of the targeting protein, it is advisable to assay this binding activity by standard means. For example, if the targeting protein is a growth factor, standard cell culture assays of the growth factor activity can be used. If the targeting

protein is a recombinant antibody, methods of measuring antibody affinity well known in the art, such as quantitative ELISA, can be used.

D. Administration of the ^{32}P-labeled Targeting Protein

Generally, the dosage of administered ^{32}P-labeled protein will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, and previous medical history. Typically, it is desirable to provide the recipient with a dosage of labeled protein which is in the range of from about 1 pg/kg to 10 mg/kg (amount of agent/body weight of patient), although a lower or higher dosage may also be administered.

For therapeutic applications, about 1-50 milligrams of ^{32}P-labeled protein will be administered, normally daily for a period of several days.

Administration of labeled proteins to a patient can be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, by perfusion through a regional catheter, or by direct intralesional injection. When administering the protein by injection, the administration may be by continuous infusion, or by single or multiple boluses.

The labeled proteins of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby labeled proteins are combined in a mixture with a pharmaceutically acceptable carrier. A composition is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient patient. Sterile phosphate-buffered saline is one example of a pharmaceutically acceptable carrier. Other suitable carriers are well-known to those in the art. See, for example, REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Ed. (1990).

For purposes of therapy, a ^{32}P-labeled protein and a pharmaceutically acceptable carrier are administered to

a patient in a therapeutically effective amount. A combination of a ^{32}P-labeled protein and a pharmaceutically acceptable carrier is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient. A targeted therapeutic agent is therapeutically effective if it delivers a higher proportion of the administered dose to the intended target than accretes at the target upon systemic administration of the equivalent untargeted agent.

To be therapeutically effective the labeled protein and carrier may need to be administered in combination with other therapeutic agents or as part of a broader treatment regimen. Physicians now are currently of the opinion that the effectiveness of targeted therapeutics can often be greatly increased when used in a combination therapy approach. For example, high-dose radioimmunotherapy for B-cell lymphomas, which causes severe hematologic toxicity when used alone, has been shown to be highly effective when used in combination with autologous bone marrow reinfusion. Press *et al.*, "Treatment of Relapsed B Cell Lymphomas with High Dose Radioimmunotherapy and Bone Marrow Transplantation" in CANCER THERAPY WITH RADIOLABELED ANTIBODIES, Goldenberg, Ed. (CRC Press, Boca Raton, 1995) ch. 17. In another example a five-fold enhancement of tumor uptake of a radiolabeled antibody is observed when the tumor is preirradiated. Leichner *et al.*, *Int. J. Radiat. Oncol. Biol. Phys.* 14:1033 (1987). Mechanisms which have been shown to have the potential for improving the clinical efficacy of radioimmunotherapy are also discussed in DeNardo *et al.*, "Overview of Obstacles and Opportunities for Radioimmunotherapy of Cancer" in CANCER THERAPY WITH RADIOLABELED ANTIBODIES, Goldenberg, Ed. (CRC Press, Boca Raton, 1995) ch. 11. Efforts to develop such combination protocols, as well as to investigate dose-limiting side

effects and to potentiate and amplify targeting, uptake, and beneficial side effects, are underway in many laboratories and hospitals and are expected to further enhance the utility of targeted therapeutic agents.

Additional pharmaceutical methods may be employed to control the duration of action of the labeled protein in a therapeutic application. Controlled release preparations can be prepared through the use of polymers to complex or adsorb a protein. For example, biocompatible polymers include matrices of poly(ethylene-co-vinyl acetate) and matrices of a polyanhydride copolymer of a stearic acid dimer and sebacic acid. Sherwood *et al.*, *Bio/Technology 10*:1446-1449 (1992). The rate of release of a ^{32}P-labeled protein from such a matrix depends upon the molecular weight of the protein, the amount of protein within the matrix, and the size of dispersed particles. Saltzman *et al.*, *Biophysical. J. 55*:163-171 (1989); and Sherwood *et al.*, *supra*. Other solid dosage forms are described in REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Ed. (1990).

Having now generally described the invention, the same will be more readily understood through reference to the following Examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE 1

Construction of F(ab)'/F(ab)'2 Bacterial Expression Vector for Humanized anti-CEA antibody MN14.

MN14 is a murine monoclonal antibody which recognizes carcinoembryonic antigen (CEA). The genes encoding the heavy and light chains of MN14 were cloned from the MN14-producing hybridoma by standard methods, and were humanized according to the method of Jones *et al.*, (*Nature* 321:522 (1986)).

(i) Cloning Strategy

A pUC19 based vector, pSW1VHpoly (Ward *et al.*, *Nature* 341:544 (1989)), was used to build the expression vector

for bacterial expression for humanized MN14. To construct the expression vector it was necessary to graft a ribosomal binding site (RBS) and a PelB sequence in-frame to the gene encoding the N-terminus of the heavy chain variable region (VH), the constant region domain 1(CH1), and the IgG1 hinge region (h). A termination codon was introduced immediately 3' to the hinge region coding sequence. Similarly, a separate RBS and PelB sequence were joined in-frame to the gene encoding the light chain variable region (VK), and kappa chain constant region (CK). The heavy and light chain fragments were ligated together as a dicistronic gene, driven by a single inducible LacZ promoter, as shown in Figure 1. To accomplish the task, several rounds of amplification by polymerase chain reaction (PCR) and multiple subcloning steps were required. The strategy is summarized in Figures 2-4.

(ii) Design of Oligonucleotide Primers for PCR and Sequencing Reactions.

Computer analysis was employed to assist in the design of primers for PCR and sequencing reactions. A total of 7 primers were required for the PCR cloning of both the heavy and light chain fragments (see Table 1). To confirm the sequences of the whole dicistronic gene (1.5 kb) encoding the F(ab')/F(ab')$_2$ fragments, 10 additional sequencing primers were required for intensive dideoxy sequencing reactions (Table 2). All primers were synthesized by Genosys Biotechnologies, Inc. (Woodlands, Texas).

(iii) Heavy Chain Fragment cDNA Synthesis and Amplification

First strand cDNA was reverse transcribed from clones secreting hMN14 by standard methods using a primer complementary to the coding sequences for the human IgG1 hinge region. In the primer, (M14H3'- see Table 1), a stop codon and three restriction sites were introduced to

prevent translation beyond the hinge region and facilitate subsequent cloning/subcloning.

To fuse the PelB sequence in-frame with VH, two different 5' primers, (M14H5'2 and M14H5'1 - see Table 1), were used in a two-step PCR reaction (Figure 2). In the first PCR, the heavy chain fragment cDNA (Fd + hinge) was subjected to 30 cycles of PCR reaction using M14H3' in conjunction with the primer M14H5'2, which contains a sequence complementary to the 5' region of VH fused in-frame to a partial sequence of PelB. The PCR product thus obtained was subjected to a second round of PCR reaction using M14H3' in conjunction with the primer M14H5'1 (see Table 1), which contains an extended but incomplete PelB sequence overlapping the 5' portion of M14H5'2 (Figure 2). A band of the expected size (~750 bp) was detected when the PCR reaction mixture was analyzed by electrophoresis through a 2% agarose gel.

(iv) Cloning and Sequencing the Gene Encoding the Heavy Chain Fragment

The PCR product produced in (iii) above contained the unique restriction sites, XbaI, EagI and BglII at its 3' end and an AlwN1 site at the 5' end of the incomplete PelB sequence. It was digested with XbaI and AlwN1, gel-purified and subcloned into the corresponding restriction sites in the vector pSW1VHpoly. The joining of the incomplete PelB sequence at the AlwN1 site with the vector reconstituted the full length sequence of PelB, fused in-frame to the heavy chain fragment. This heavy chain staging vector is named as MN14HpSW1.

Three bacterial clones containing inserts of the correct size were subjected to sequencing reactions. A frame-shift error resulting from a single nucleotide deletion was noted in one of the clones. The DNA sequences for the rest of the clones were identical to the sequence designed originally.

(v) Subcloning of the PelB Gene into VKpBR

A different approach to that used for the heavy chain was adopted to introduce a separate RBS and a full PelB sequence fused in-frame with the VK domain. A PCR reaction employing PelBL3' and PelBL5' as the primers (Table 1) and pSW1VHpoly as the template was carried out to amplify the RBS and PelB containing sequence (Figure 3). The primer PelBL3' contains a partial 5' VK sequence fused in-frame with a region complementary to the 3' end of the PelB gene. The partial VK sequence in PelBL3' contains a PvuII site for in-frame ligation to the full length light chain gene. A PCR product of the expected size (~ 120 bp) was obtained and subcloned into the BamHI/PvuII site of VKpBR. VKpBR is a staging vector derived from pBR327 (Leung *et al., Hybridoma, in press* (1994)). It contains a 600 bp fragment inserted between the HindIII and BamH1 sites of pBR327, containing the IgG promoter, an antibody leader sequence and a variable chain kappa sequence. This RBS and PelB containing vector, designated PelBpBR, served as the staging vector for subcloning the light chain gene.

(vi) Kappa Chain cDNA Synthesis and Amplification

First strand cDNA was synthesized by standard methods using M14KFOR (Table 1), an oligonucleotide complementary to the 3' end of the kappa constant region.

PCR amplifications were carried out using the primer set, M14KBACK and M14KFOR (Figure 3). A product of the expected size (~750 bp) was identified. Primer M14KBACK contained a PvuII site which, when fused into the vector, PelBpBR, at corresponding restriction sites, reconstituted a full length Kappa chain attached in-frame to the PelB signal sequence.

(vii) Cloning and Sequencing of Kappa Chain DNA

The kappa chain PCR product (~750 bp) was digested with PvuII & XbaI and subcloned into the corresponding restriction sites of PelBpBR (Figure 3), forming the complete light chain vector, M14LpBR. This contained a

5' RBS sequence and a PelB gene fused in-frame with the full cDNA of humanized MN14 kappa light chain. Three of the bacterial clones containing inserts of the anticipated size were subjected to intense sequencing reactions. All the sequences were found to be identical to the original design.

(viii) Combining Heavy and Light Chain Fragments to Form the Final Expression Vector

To construct the dicistronic F(ab')/F(ab')$_2$ bacterial expression vector, the RBS and PelB-containing light chain sequences were joined downstream of the heavy chain sequence in the vector MN14pHpSW1. The light chain sequence containing the RBS and pelB gene was excised from M14LpBR by digestion with BglII and XbaI. It was then ligated into the corresponding restriction sites in the heavy chain vector, MN14HpSW1, to form the final bacterial expression vector for the humanized MN14 F(ab')/F(ab')$_2$, MN14pBEV (Figures 1 and 4).

(ix) Sequencing of the Bacterial Expression Vector MN14pBEV

Ten additional sequencing primers (Table 2) were used to determine the full length sequence of the segment containing the light and heavy chains together with their respective RBSs and PelB genes in MN14pBEV (Figure 5). The sequences were found to contain no undesirable mutations.

EXAMPLE 2

Mutation of MN14pBEV to Incorporate a Protein Kinase Substrate Sequence

The PCR was used to introduce a 21-nucleotide sequence at the 3' terminus of the heavy chain portion of the dicistronic gene contained in MN14pBEV. This sequence encoded the 7 amino acid peptide WRRASLG, which is a substrate for PKA, and is derived from the peptide LRRASLG, which has the trivial name "Kemptide".

A 55-nucleotide oligomer (H-KPT in Figure 6) was used as a primer for the PCR. This nucleotide contains a termination codon and a *Bgl*II restriction site at a region corresponding to the C-terminus of the peptide. It also encodes a derivative of the Kemptide sequence and the last 7 amino acids of the hinge region of the heavy chain of MN14. H-KPT was used together with a primer derived from the CH1 region of MN14 (H5'BX in Figure 6) to amplify a ~100 bp fragment using MN14pBEV as a template. The resulting PCR product encoded part of the CH1 region fused in-frame to the Kemptide. It also contained a *BstX*1 site at its 5' end and a *Bgl*II site at its 3' end. The PCR product was digested with *BstX*1 and *Bgl*II and ligated into the corresponding restriction sites within MN14pBEV. Figure 7 shows a schematic map of the resultant expression vector, designated hMN14 Fab-KPT.

EXAMPLE 3

Expression and Purification of the MN14-Kemptide Fusion Protein

To express the fusion protein in bacteria, hMN14 Fab-KPT was transformed into the E. coli BMH71-18 mutS (Clontech, Palo Alto, CA). Transformants were grown overnight at 37°C in 2xTY (for 1 liter: 16g Bactotryptone, 10g yeast extract, 5g NaCl) in the presence of 55mM glucose and 100 μg/ml of Ampicillin. The overnight culture was pelleted, washed twice with 2xTY or LB, and resuspended with pre-warmed 2xTY in the presence of 1-5 mM isopropylthiogalactoside (IPTG). The induction was allowed to proceed for 20-24 at 37°C. *E. coli* were removed by centrifugation and the proteins in the cell culture medium were purified on a QuickMab column (Sterogene, Arcadia, CA) and concentrated using a Centricon 30 spin column (Amicon, Beverley, MA). The proteins thus obtained were compared to IgG-derived hMN14 $F(ab')_2$ and hMN14 Fab' fragments by SDS-PAGE analysis, under both reducing and non-reducing conditions. The gel

showed that the hMN14 Fab-kemptide fusion protein contained both $F(ab')_2$ and Fab' fragments. Under non-reducing conditions, the fusion protein $F(ab')_2$ fragment migrated at an almost identical apparent molecular weight to the IgG-derived $F(ab')_2$, whereas the fusion protein Fab' migrated at a slightly smaller apparent molecular weight than the major band in the IgG derived Fab' material. Under reducing conditions the fusion protein migrated at a smaller apparent molecular weight than the main band in the IgG-derived material.

EXAMPLE 4

Phosphorylation of the Fusion Protein produced from hMN14 Fab-KPT.

Before phosphorylation, the fusion protein from hMN14 Fab-KPT was further purified on a centrifuged size Sephadex G-50-80 exclusion column (Pharmacia, Piscataway, NJ) using 0.1M sodium phosphate, pH 7. The final concentration of protein was ~1 mg/ml. To a solution of 6.3 μl of enzyme buffer (250 mM sodium phosphate, 25 mM $MgCl_2$, 1.25 mM EGTA) was added 1 μl of $[^{32}P]\gamma ATP$ (15.4μCi), followed by 25 μl of the hMN14 Fab-KPT protein solution and 5 μl (25U) bovine heart protein kinase (Sigma). The reaction mixture was incubated at 37°C and monitored on size exclusion HPLC, using a BioSil 250 column (Biorad, Hercules, CA) eluting with 0.2M sodium phosphate, pH 6.8 at 1 ml/min. After 30 minutes, HPLC showed 68.9% phosphate incorporation, and after 60 minutes, 77.6% incorporation. The phosphorylated protein was purified by passage through a Sephadex G-50-80 spin column. Figures 8 and 9 show the HPLC results.

Figure 8 shows HPLC analyses of IgG-derived hMN14Fab' and Fab'-kemptide fusion protein before and after labeling with ^{32}P. The HPLC eluate was monitored either at 280 nm (panels A,B,C) or by radiation detector (panels D,E): (A) shows hMN14 Fab' kemptide fusion protein; (B) shows IgG- derived hMN14Fab'; (C) shows IgG-derived hMN14 $F(ab')_2$; (D) shows $[\gamma]^{32}P$-ATP; (E) shows hMN14 Fab'-

kemptide fusion protein after ^{32}P labeling with bovine heart protein kinase. Figure 9 shows HPLC analyses before (A) and after (B) reacting with soluble CEA.

EXAMPLE 5

Immunoreactivity of the Phosphorylated hMN14 Fab-KPT

The phosphorylated hMN14 Fab-KPT was reacted with soluble CEA, and the reaction mixture analyzed by SDS-PAGE under non-denaturing conditions. Briefly, 0.2 μCi (~0.3 μg) of ^{32}P labeled hMN14-KPT, either alone or mixed with 24 μg (80-fold excess) of CEA in PBS in a final volume of 10 μl of 2x loading buffer [20% (v/v) glycerol, 0.125 M Tris.HCl (pH 6.8)] was analyzed in a 4-20% SDS-PAGE gel (Integrated Separation Systems, Natick, MA). In the absence of the CEA the fusion protein appears as a band with an apparent molecular weight of approximately 35 kDa. In the presence of the CEA the 35 kDa band disappears and a band appears at an apparent molecular weight of approximately 140 kDa. These results show that the phosphorylated protein retained its ability to bind to CEA.

Immunoreactivity was also demonstrated in a blocking assay. Blocking activity of phosphorylated hMN14 Fab-KPT was compared to that of the unphosphorylated hMN14 Fab-KPT and the hMN14 Fab' fragment derived from whole hMN14 IgG. To ELISA plates coated with an anti-CEA IgG, MN-5 (Immunomedics, Morris Plains, NJ), CEA (Calbiochem, San Diego, CA) at a concentration of 50 ng/ml was added, and the plate was incubated for 1h at 20°C. Horseradish peroxidase-conjugated murine MN-14, at a concentration of 200 ng/ml, was mixed with varying concentrations of control antibodies, as well as unlabeled or ^{32}P-labeled hMN14-KPT. The mixture was incubated for 1h at 20°C. The amount of peroxidase-conjugated MN-14 bound was revealed with a substrate solution containing 0.0125% H_2O_2 and 0.004 M *o*-phenylenediamine dihydrochloride. Figure 10 shows the results obtained, demonstrating again that phosphorylated hMN14 Fab-KPT protein retained full

binding activity against CEA. In Figure 10 IgG-derived hMN14 Fab' is shown by closed squares, hMN14 Fab'-kemptide fusion protein by open circles, and ^{32}P-labeled hMN14 Fab'-kemptide fusion protein by closed circles.

EXAMPLE 6

Construction of a gene encoding an Interleukin 6 - Kemptide Fusion Protein

A gene encoding interleukin 6 (IL-6) is purchased from R&D Systems (Minneapolis, MN, catalogue #BBG 17). Two oligonucleotide primers are synthesized for PCR amplification and mutagenesis of this gene. The first primer contains the 5' sequence of the gene, but introduces an *Nde*1 restriction site at the translation initiation site. The second primer is complementary to the 3' end of the gene, but also includes a triplet repeat of a sequence encoding the Kemptide amino acid sequence and a stop codon, both in frame with the IL-6 gene. This second primer also introduces a *Hind*III restriction site 3' of the Kemptide sequence to allow cloning of the PCR product.

After PCR amplification using AmpliTaq™ polymerase (Perkin-Elmer) under the standard conditions recommended by the manufacturer, the amplification products are purified using a GeneClean™ kit (Bio 101, San Diego, CA), digested with *Nde*1 and *Hind*III, and ligated into the pProEX-1 vector (Life Technologies, Gaithersburg, MD). This vector introduces a DNA sequence encoding a 6-histidine motif at the 5' end of the gene, linked by a sequence encoding a cleavage site for tobacco etch virus (TEV) protease. The plasmid is transformed into DH10B *E. coli* cells (Life Technologies), and plated out overnight on agar plates containing 100 μg/ml ampicillin. Three single colonies are picked for sequences analysis and shown to contain the desired gene sequence with no frame-shifts or other mutations.

EXAMPLE 7

Expression and Purification of the IL-6-Kemptide Fusion Protein

Single colonies of the transformed bacteria from Example 6 are inoculated into 10 ml LB medium containing 100 μg/ml ampicillin, and grown overnight at 37°C. 8 ml of this culture is used to inoculate 800 ml of LB/ampicillin, and the culture is incubated at 37°C until an A_{590} of 0.6 is reached. IPTG is added to a final concentration of 0.6 mM and the culture reincubated at 37°C for 3 hours. The cells are then collected by centrifugation.

The cell pellet is suspended in 50 mM Tris-HCl (pH 8.5), 10 mM 2-mercaptoethanol, and 1 mM PMSF to a concentration of 4 ml buffer per gram of cells (wet weight). The suspension is sonicated to greater than 60% lysis (lysis is monitored by observing samples of the sonicate microscopically), and cell debris removed by centrifugation. The supernatant is applied to a 2 ml column of Ni-NTA resin (Qiagen), equilibrated with buffer A (20 mM Tris-HCl, pH 8.5; 100mM KCl; 10 mM 2-mercaptoethanol; 10% glycerol). The column is washed with 10 column volumes of buffer A followed by 2 column volumes of buffer B (same as buffer A except KCl is 1 M) and 2 more column volumes of buffer A. The column is then eluted with buffer A to which imidazole has been added to 100 mM. Column fractions are monitored by SDS-PAGE.

Fractions containing the desired IL-6-Kemptide fusion protein are pooled and dialyzed against 100 volumes of 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 1 mM DTT. The solution is incubated at 30°C and recombinant TEV protease is added to cleave off the 6 histidine motif. The cleavage is monitored by SDS-PAGE. When the cleavage is complete the reaction mixture is purified on a Ni-NTA column as above. The by-products and protease are retained on the column, affording pure IL-6-Kemptide fusion protein.

Biological activity of the fusion protein is assayed by measuring the concentration of the protein required to cause half-maximal thymidine incorporation into B9 hybridoma cells (ATCC, Rockville, MD). Comparison with a standard preparation of human IL-6 (Life Technologies) demonstrates that the fusion protein retains full biological activity.

EXAMPLE 8

Phosphorylation of the IL-6-Kemptide fusion protein.

The fusion protein from Example 7 is dialyzed into 0.1M sodium phosphate, pH 7. The final concentration of protein is adjusted to ~1 mg/ml. To a solution of 6.3 μl of enzyme buffer (250 mM sodium phosphate, 25 mM $MgCl_2$, 1.25 mM EGTA) is added 1 μl of [^{32}P]γATP (15.4μCi), followed by 25 μl of the IL-6-Kemptide fusion protein solution and 5 μl (25U) bovine heart protein kinase (Sigma). The reaction mixture is incubated at 37°C and monitored on size exclusion HPLC, as in Example 4. The phosphorylated protein is purified by passage through a Sephadex G-50-80 spin column. The phosphorylation reaction is also carried out under identical condition using cold ATP, and the biological activity of the phosphorylated product assayed as in Example 7. This shows that phosphorylation causes no diminution in IL-6 activity of the fusion protein.

Table 1

SEQUENCES OF PRIMERS USED FOR THE PCR CLONING OF HUMN14.F(AB')/F(AB')2 FRAGMENTS

M14H5'1

5' *AlwnI*
GGCAAGCTTCAGCCGCTGGCTTGTTGTTACTAGCAGCTCAACCTGCAATG 3'

M14H5'2

5'
GCAGCTCAACCTGCCATGGCCGAGGTCCAACTGGTGGAGAGC 3'

M14H3'

5' *XbaI* *EagI* *BglII*
GAATCTAGA CGGCCG AGATCTTTATGGGCACGGTGGGCATGTGT 3'

PelBL5'

5' *BglII*
TTAATTAACGGCCGAGATCTGCATGCAAATTCTATTTCAA 3'

PelBL3'

5' *XbaI* *PvuII*
CGAGGATCCTCTAGA CAGCTGGATGTCGGCCATCGCTGGTTGGGCAGC 3'

M14KBACK

5' *PvuII*
CACTCCGACATCCAGCTGACCCAGAGC 3'

M14KFOR

5' *XbaI*
GGTCTGAGTCTAGACCTTTAACACTCTCCCCTGTTGAAGCT 3'

Table 2

A LIST OF ADDITIONAL PIMERS USED TO ELUCIDATE THE FULL LENGTHE SEQUENCE OF THE DNA ENCODING HUMN14.F(AB')/F(AB')2 FRAGMENTS

MHSEQ1

5' CAGGTGCCTGTCTCACCCAAC 3'

MHSEQ2

5' AACTATGCGCCGTCTCTA 3'

MHSEQ3

5' CAGCCTCCACCAAGGGCCCA 3'

MHSEQ4

5' TGGGCCCTTGGTGGAGGCTGA 3'

MHSEQ5

5' ACCAGCGGCGTGCACACC 3'

MKSEQ1

5' GACATCCAGCTGACCCAGAGC 3'

MKSEQ2

5' CTGGTACCAAGCTACAGAAGT 3'

MKSEQ3

5' ACTGTGGCTGCACCATCTGTC 3'

MKSEQ4

5' CCAGATTTCAACTGCTCATC 3'

MKSEQ5

5' CAGCAAGGACAGCACCTACA 3'

What Is Claimed Is:

1. A protein capable of binding specifically to a complementary molecular species by virtue of a complementarity-determining region thereof, said protein further comprising a heterologous peptide sequence that is a substrate for a protein kinase, whereby said protein can be radiolabeled with ^{32}P or ^{33}P.

2. The protein of claim 1 which is a fusion protein comprising a peptide sequence including said complementarity-determining region and a peptide sequence comprising said substrate.

3. The protein of claim 1 wherein said substrate peptide sequence is a substrate for at least one protein kinase chosen from the group consisting of serine/threonine-specific, and tyrosine-specific protein kinases.

4. The protein of claim 3, wherein said substrate peptide sequence is a substrate for at least one protein kinase chosen from the group consisting of: bovine heart protein kinase, protein kinase A, protein kinase C, calcium/calmodulin-dependent protein kinase II, casein kinase II, phosphorylase kinase, EGF-receptor kinase, insulin receptor kinase, *src*, *abl*, *lck*, *fyn*, *yes*, and $p72^{syk}$.

5. The protein of claim 1, wherein said peptide sequence is selected from the group comprising Leu-Arg-Arg-Ala-Ser-Leu-Gly and Trp-Arg-Arg-Ala-Ser-Leu-Gly.

6. The protein of claim 1, wherein said peptide sequence comprises Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val.

7. The protein of claim 1, wherein said peptide sequence comprises Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu.

8. The protein of claim 1, wherein said peptide sequence comprises Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys.

9. The protein of claim 1, wherein said peptide sequence comprises Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly.

10. The protein of claim 1, wherein said peptide sequence comprises Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys.

11. The protein of claim 1, wherein said peptide sequence comprises Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu.

12. An isolated DNA molecule encoding a protein according to claim 1.

13. A method of producing a protein comprising cloning the isolated DNA molecule of claim 12.

14. The method of claim 13, further comprising cloning said DNA molecule into an expression vector.

15. The method of claim 14, further comprising the step of transforming host cells with said expression vector, and recovering said transformed host cells.

16. The method of claim 15, further comprising the step of culturing said transformed host cells and recovering said protein from said cultured host cells.

17. A method of producing a ^{32}P- or ^{33}P-labelled protein comprising contacting a protein according to claim 1 with a ^{32}P- or ^{33}P-labelled phosphate or thiophosphate donor in the presence of a protein kinase which has activity for said peptide sequence.

18. The method of claim 17 wherein the ^{32}P- or ^{33}P-labelled phosphate donor is selected from $\gamma^{32}P$-ATP, γthio-$\gamma^{32}P$-ATP, $\gamma^{33}P$-ATP, or γthio-$\gamma^{33}P$-ATP.

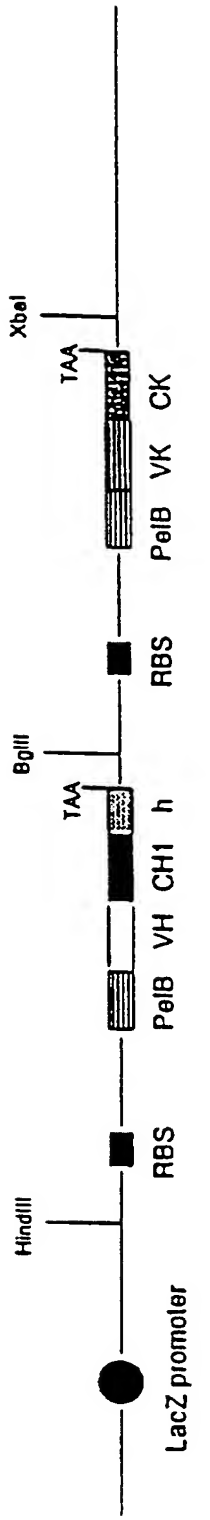
19. A kit for producing a ^{32}P- or ^{33}P- radiolabeled protein, comprising a protein according to claim 1 and a protein kinase which acts on a specific substrate peptide sequence in said protein.

20. In a method of radiotherapy of a patient suffering from a tumor or an infectious lesion, wherein a protein that specifically binds to a complementary molecule or structure produced by or associated with a tumor or an infectious lesion, and radiolabeled with ^{32}P or ^{33}P, is parenterally injected into a human patient suffering from said tumor or infectious lesion,

the improvement wherein said radiolabeled protein is a radiolabeled protein according to claim 1.

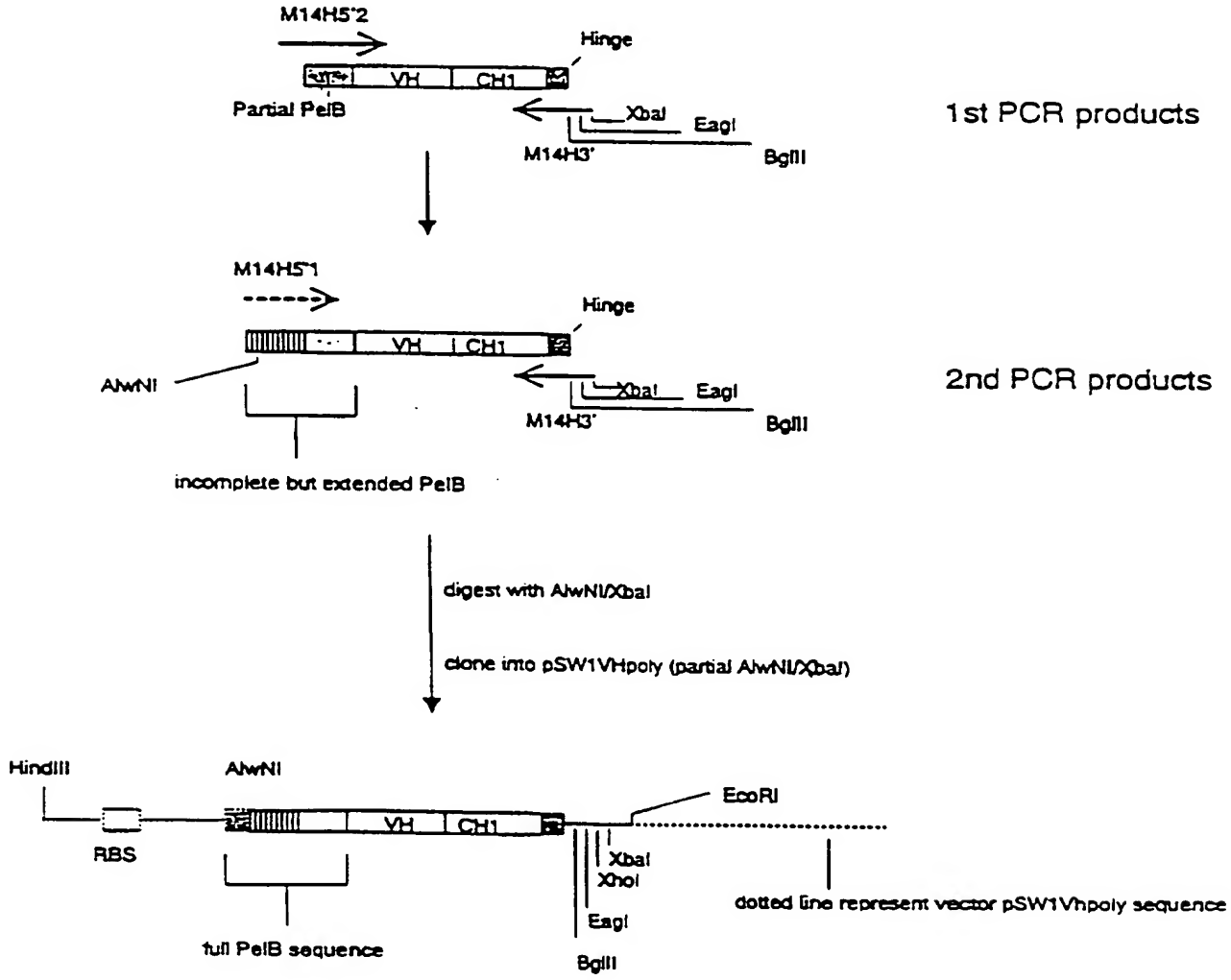
21. A pharmaceutical composition, comprising an effective amount of a ^{32}P- or ^{33}P-labeled protein produced according to claim 17, in a pharmaceutically acceptable sterile vehicle.

FIG. 1



MN14pBEV

FIG. 2



MN14HpSW1

FIG. 3

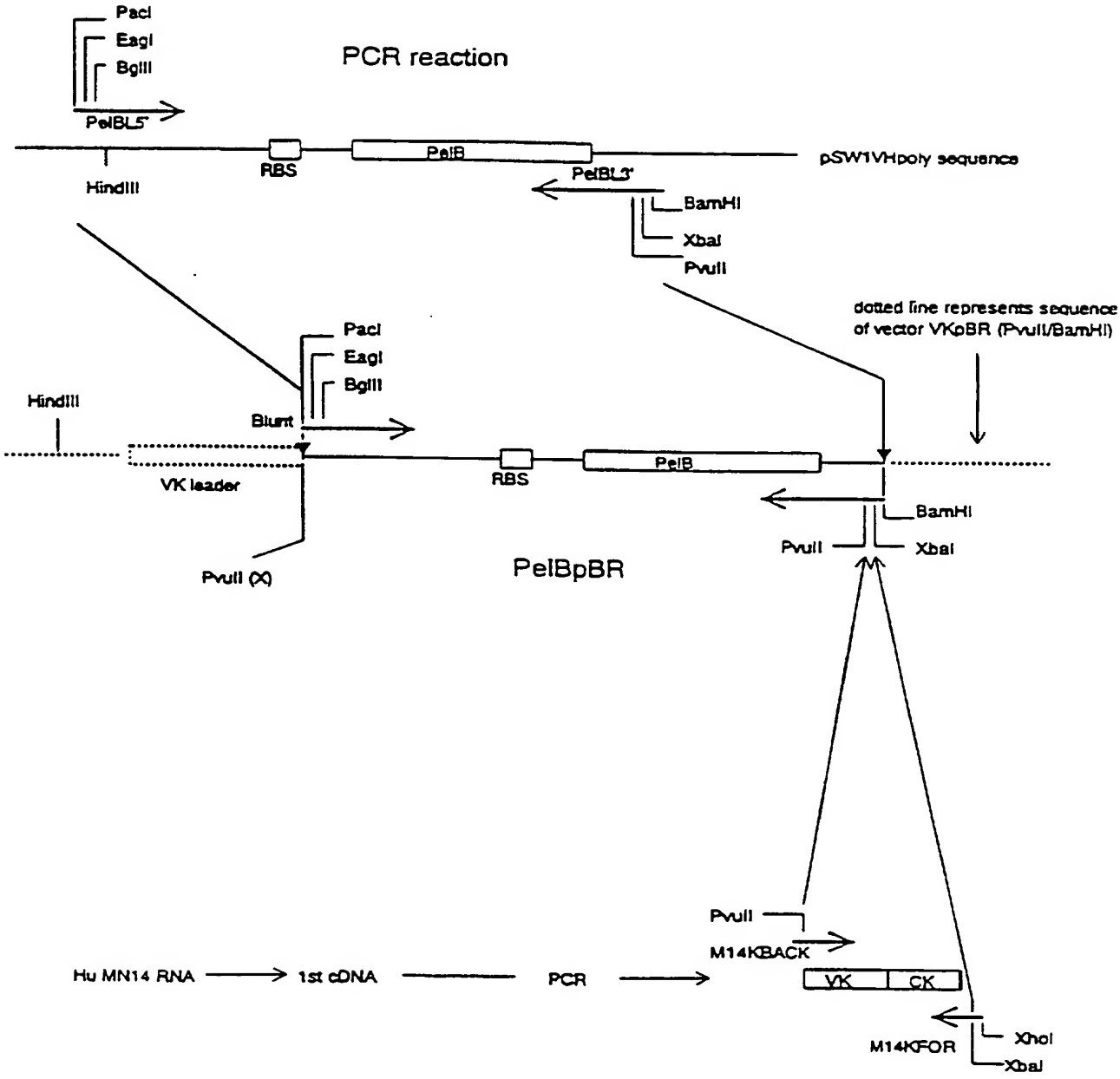


FIG. 4

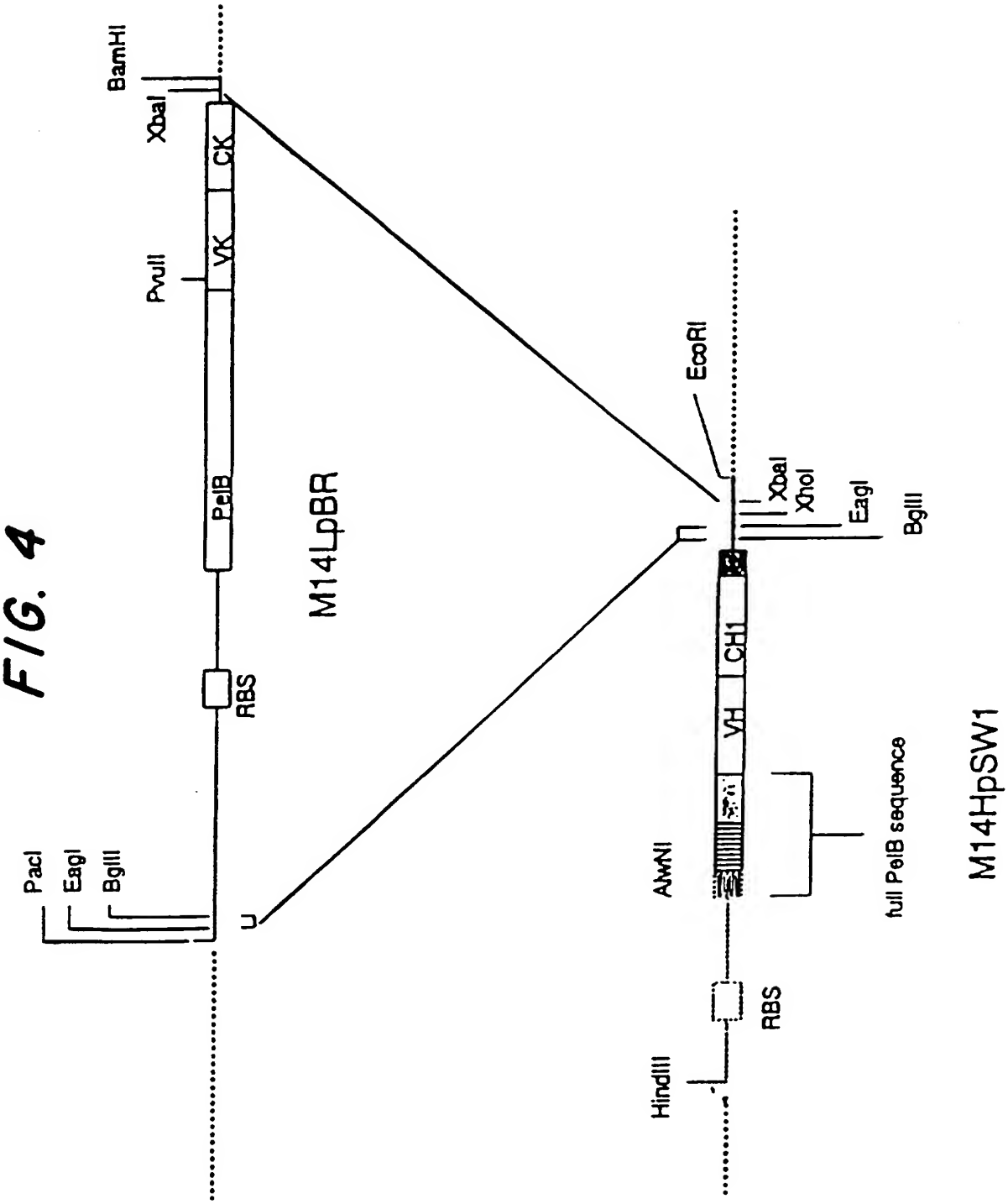
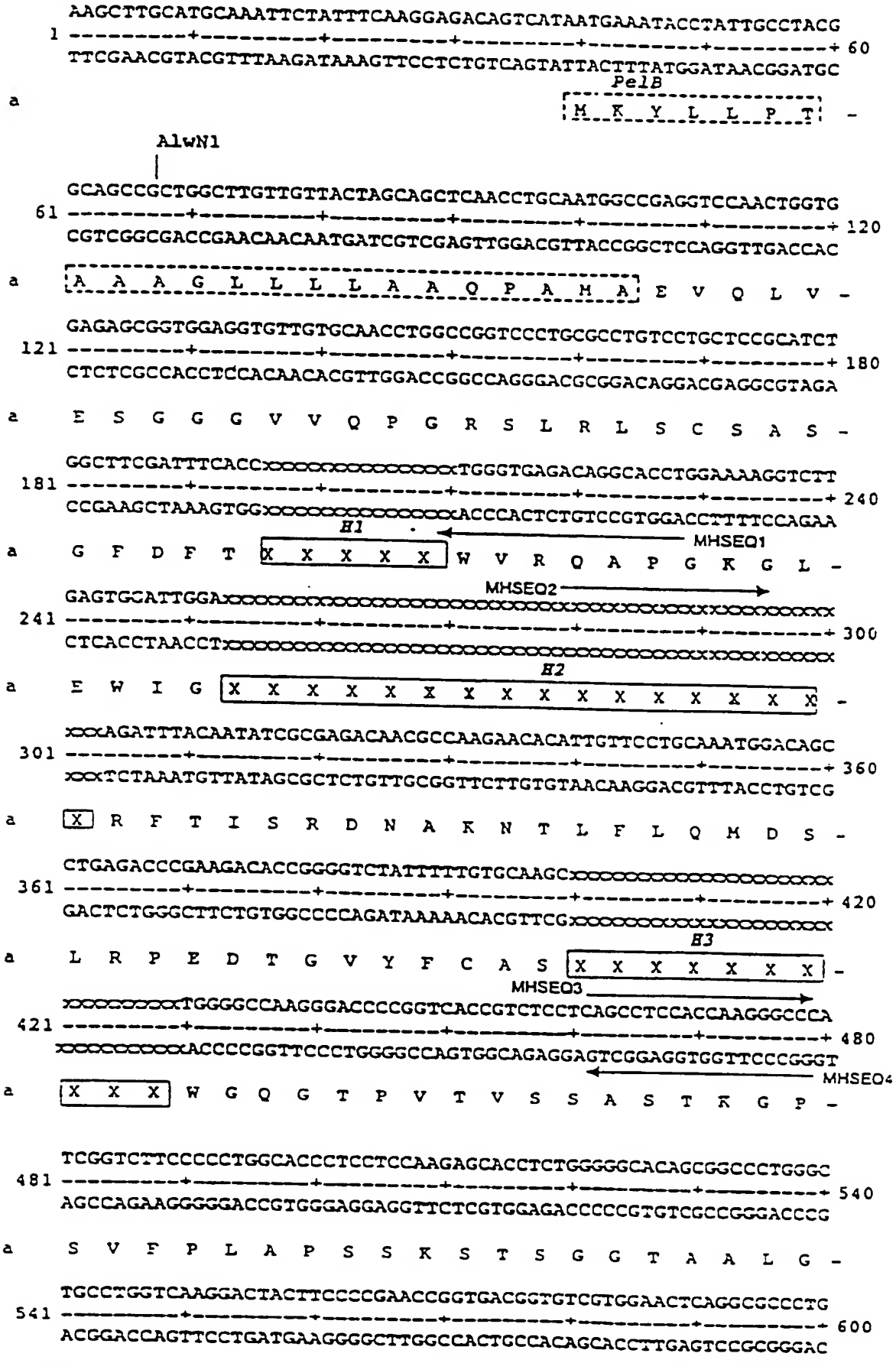


FIG. 5A



6/12

FIG. 5B

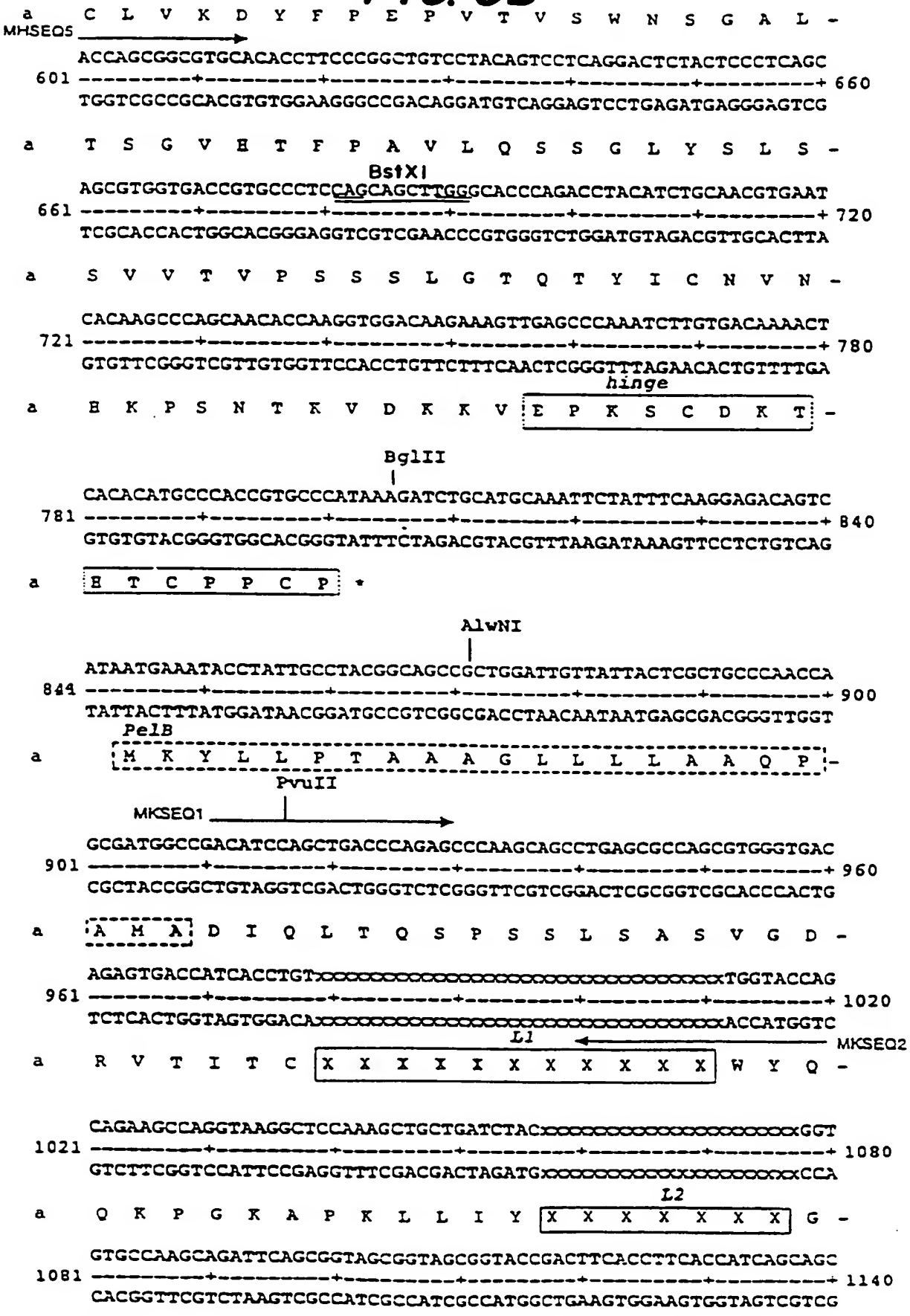


FIG. 5C

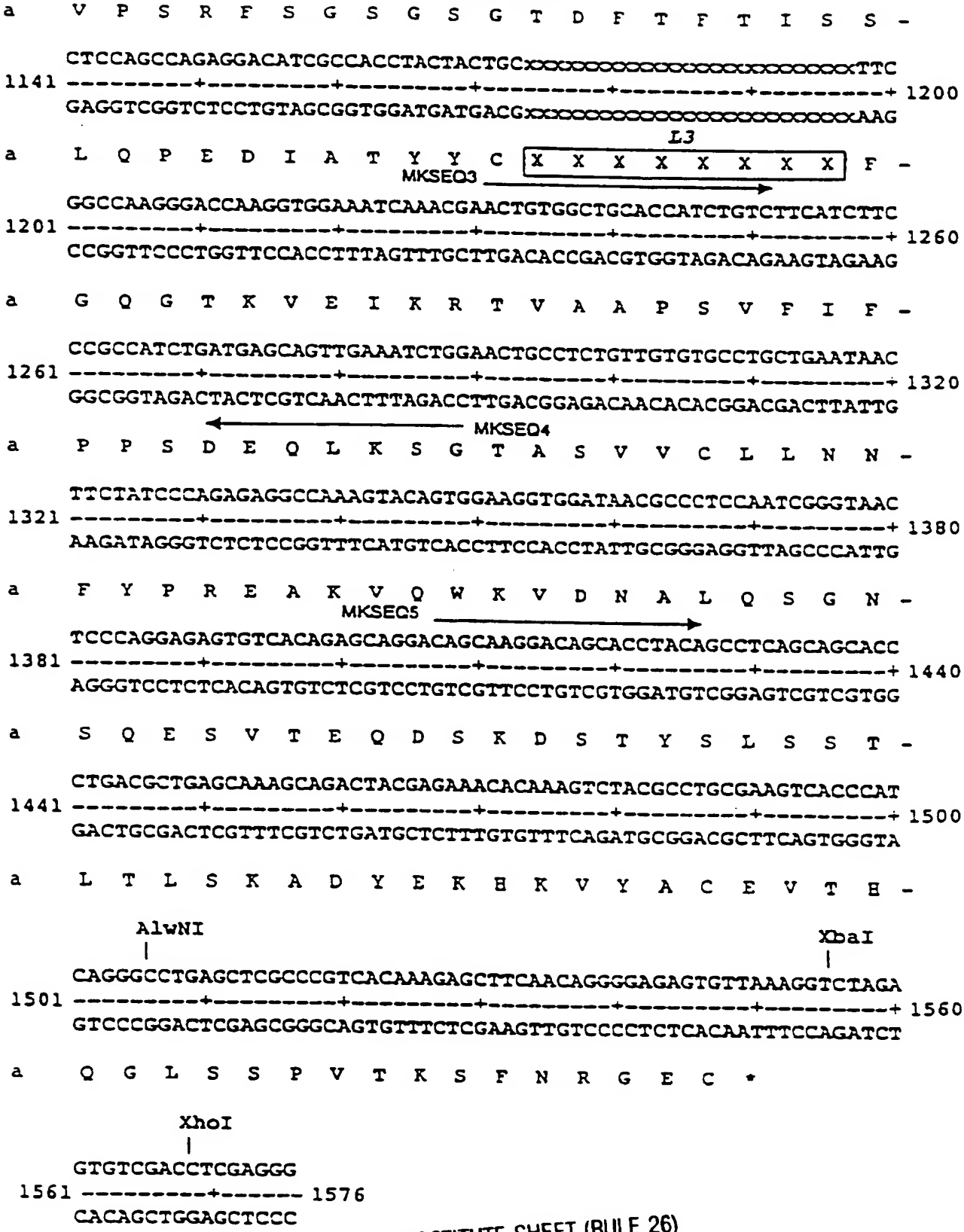


FIG. 6

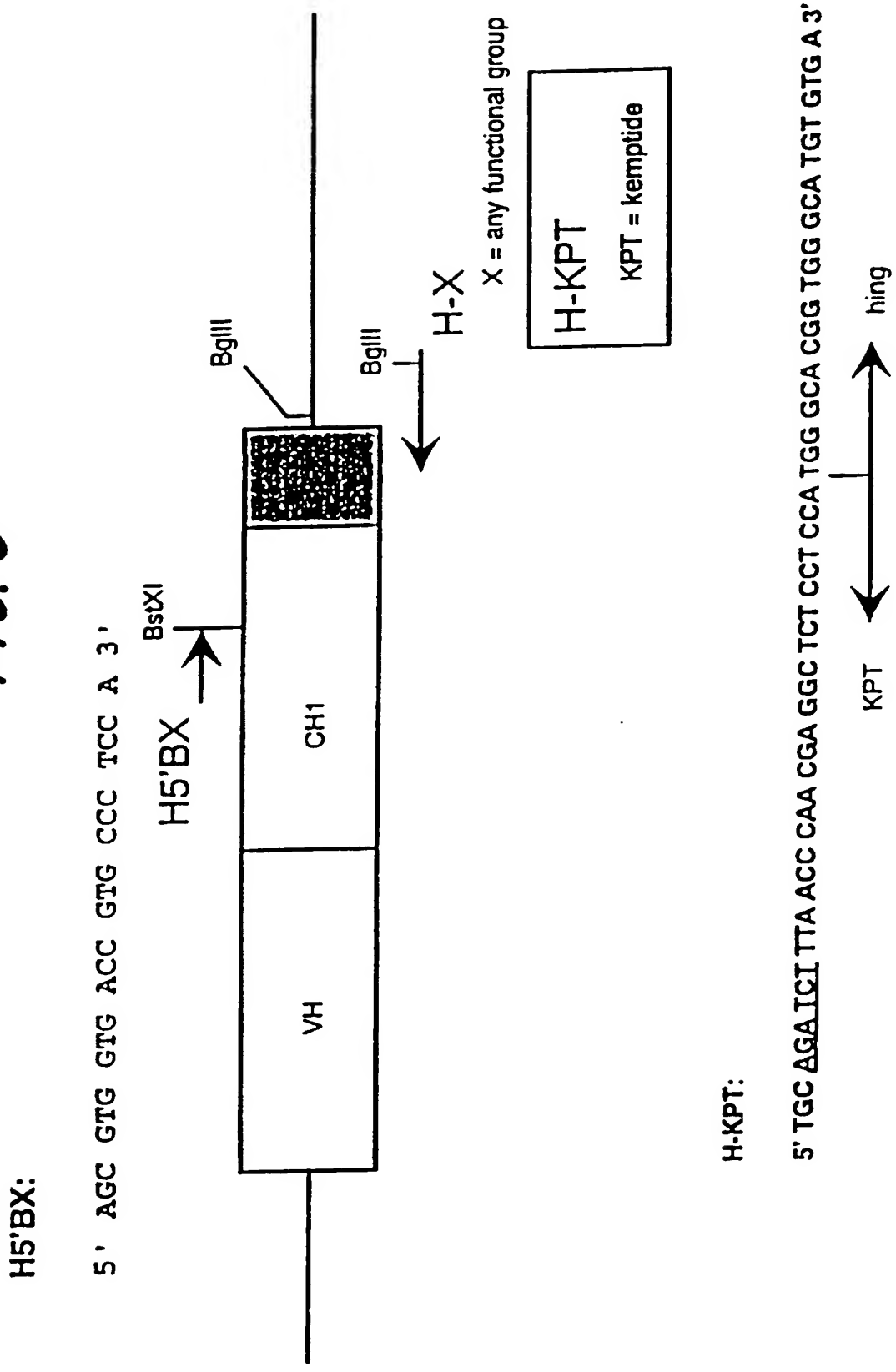


FIG. 7

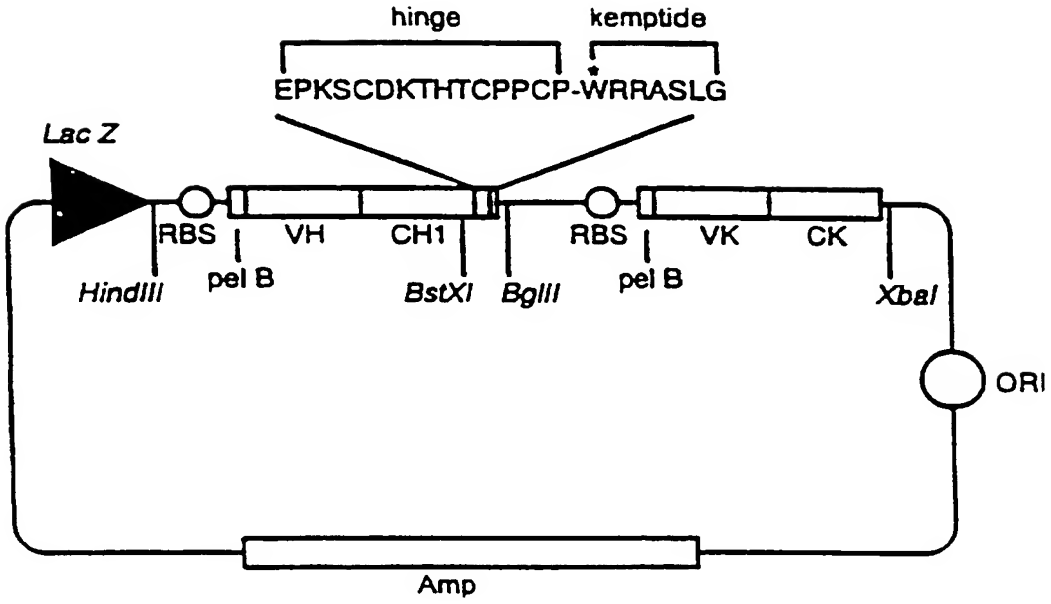


FIG. 8

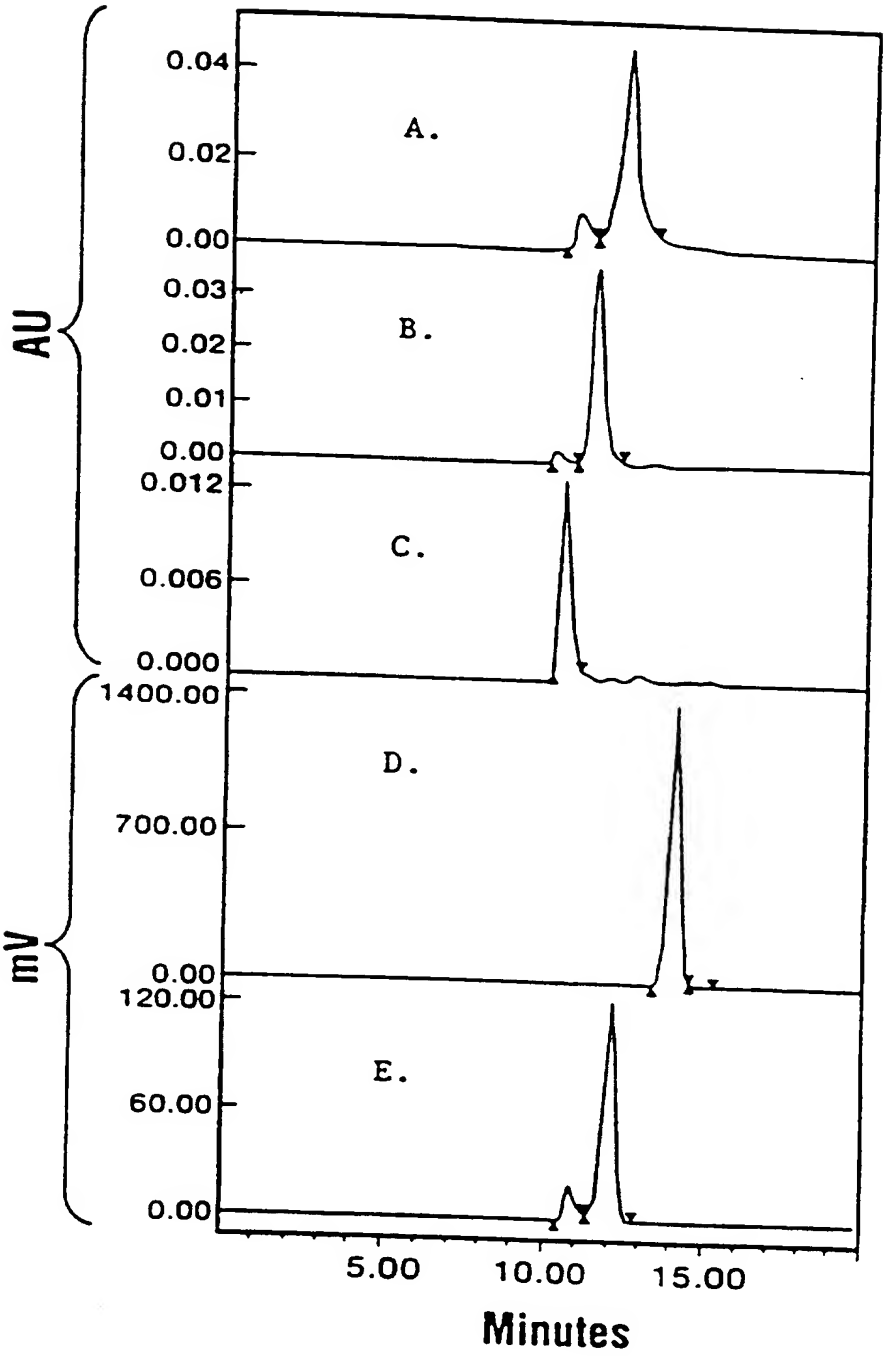
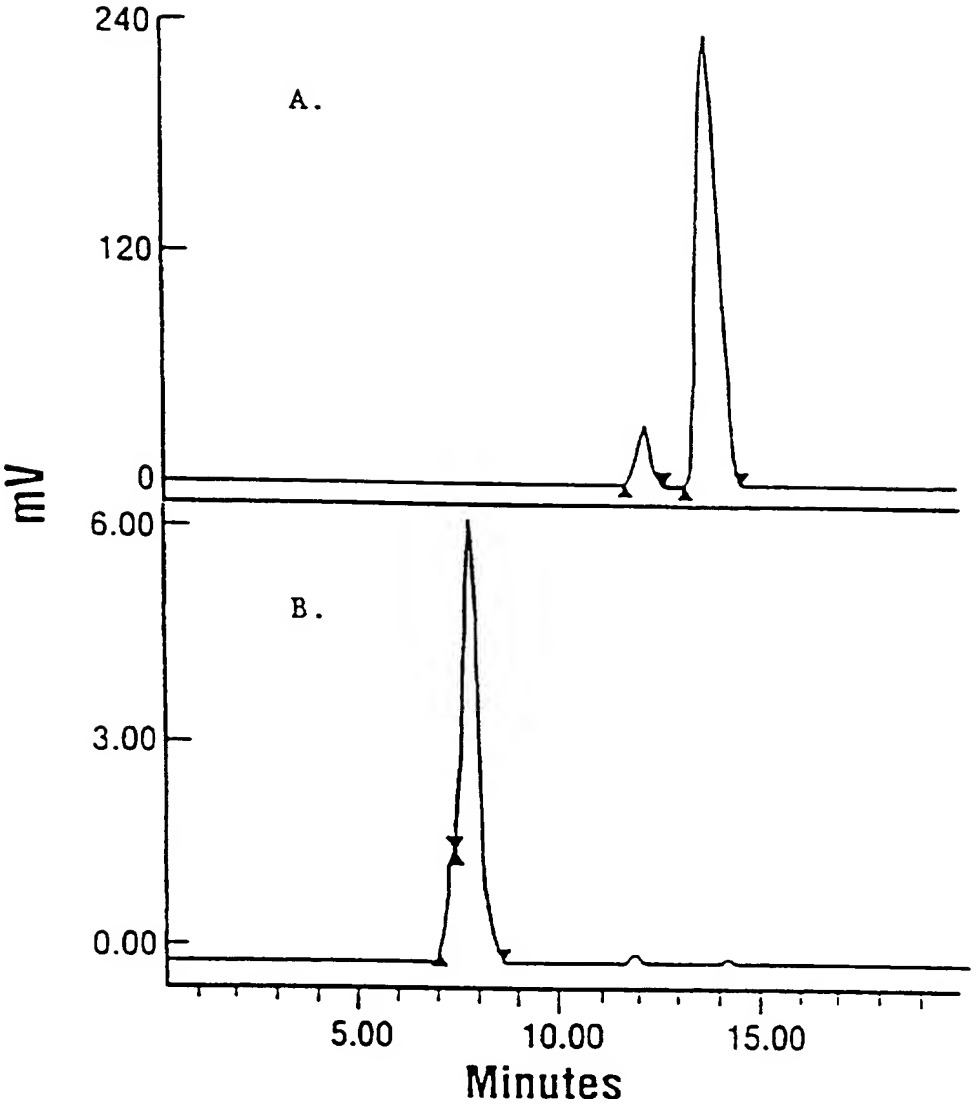


FIG. 9



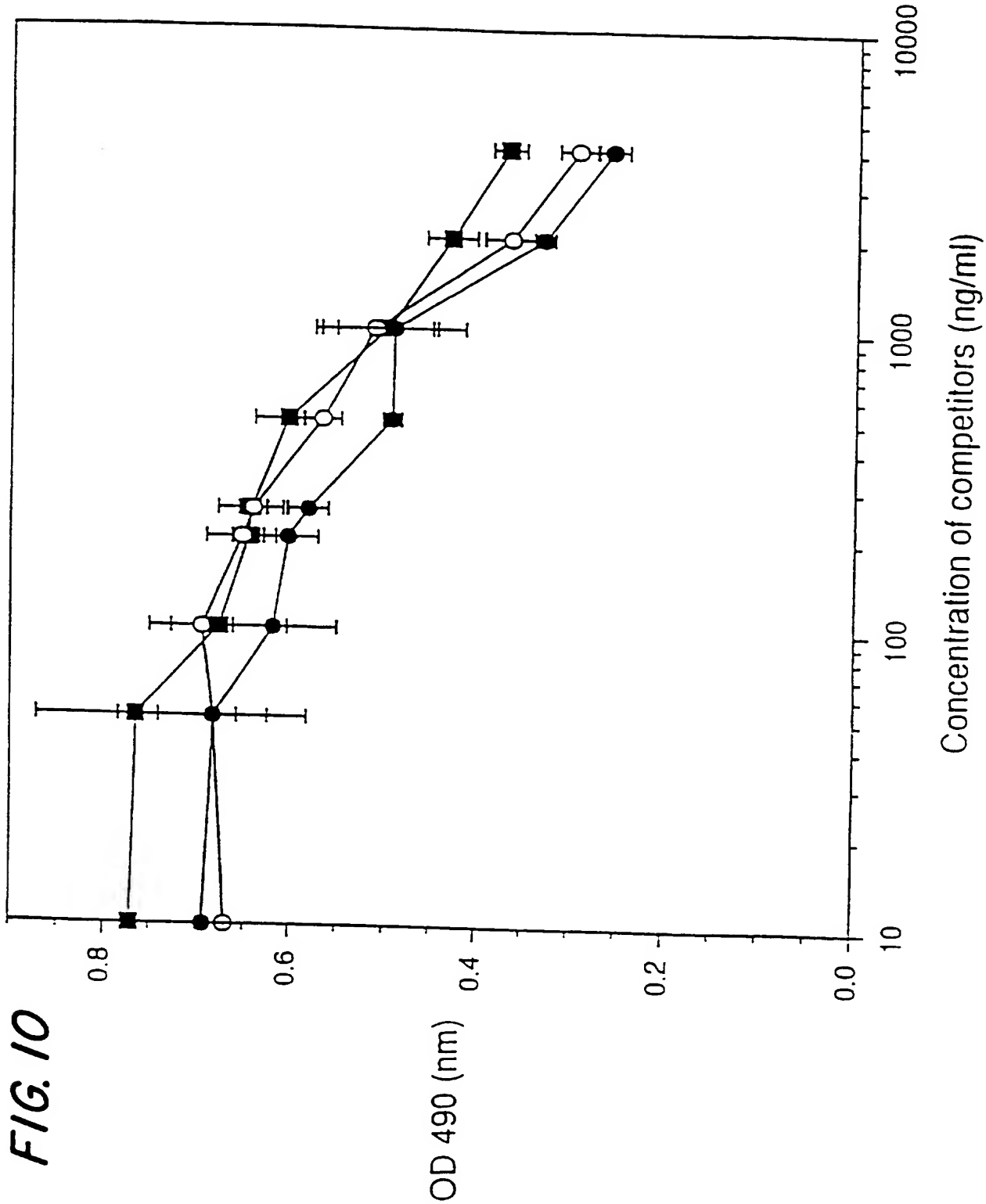


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11405

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07K 2/00, 4/00, 7/00
US CL :424/1.69, 9.6; 530/300
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/1.69, 9.6; 530/300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
journals

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAS ONLINE
search terms: protein(s), radiolabel, phorphorus, kinase, protein kinase, antibody

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	British Journal of Cancer, Volume 57, issued 1988, Foxwell et al, "Conjugation of monoclonal antibodies to a synthetic peptide substrate for protein kinase: A method for labelling antibodies with 32P", pages 489-493, see the entire document.	1-5,17-18
X	GB, A, 2,186,579 (FOXWELL ET AL) 19 August 1987, see entire document, especially, abstract, examples, page 1 (lines 127-130), page 2 (lines 1-3), page 3 (lines 14-19), and page 5 (claim 4)	1-5,17-18,21
X	WO, A, 90/11289 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 04 October 1990, see the entire document, especially, examples and pages 8-9.	1-5,17-18,20-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
06 DECEMBER 1995

Date of mailing of the international search report
28 DEC 1995

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BNSDOCID: <WO 9608506A1_I_>

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11405

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,923,802 (GALLIS) 08 May 1990, see entire document, especially, abstract, column 6 (lines 59-68), column 7 (lines 1-9 and 55-59), column 8 (lines 11-18), column 10 (lines 15-23), and columns 12-14 (claims 8-13 and 18-19).	1-5,12-19
Y,P	US, A, 5,366,889 (MACDONALD ET AL) 22 November 1994, column 8 (lines 24-55) and columns 23-24 (examples 1-2).	12-15
A	US, A, 5,212,072 (BLALOCK ET AL) 18 May 1993, see entire document.	12-16
A	Second Messengers and Phosphoproteins, Volume 14, No. 1 & 2, issued 1992, Nemani et al, "Measurement of protein phosphatase activity in biological samples using synthetic phosphopeptides", pages 65-76, see entire document.	
Y,P	US, A, 5,389,527 (BEAVO ET AL) 14 February 1995, see entire document, especially, abstract, column 4 (lines 52-68), column 5 (lines 1-54), column 6 (lines 19-38).	1-4,12-18
Y	Analytical Biochemistry, Volume 197, issued 1991, Sullivan et al, "A manual sequencing method for identification of phosphorylated amino acids in phosphopeptides", pages 65-68, see pages 65-66 (Materials and Methods).	1-5
Y	WO, A, 91/01305 (UNIVERSITY OF WALES COLLEGE OF MEDICINE) 07 February 1991, see the entire document.	1-5,12-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11405

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ **Claims Nos.:**
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ **Claims Nos.:**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ **Claims Nos.:**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Form PCT/ISA/206 Previously Mailed.)
Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☒ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11405

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-11, 13-19, and 21, drawn protein, method of producing protein, and use of protein in radiotherapy.

Group I: The following species have different structures and are expected to behave differently:

a. the first protein sequence found in Claim 5

b. the second protein sequence found in Claim 5

c. the protein sequence found in Claim 6

d. the protein sequence found in Claim 7

e. the protein sequence found in Claim 8

f. the protein sequence found in Claim 9

g. the protein sequence found in Claim 10

h. the protein sequence found in Claim 11

II. Claim 12, drawn to a DNA molecule encoding binding protein.

III. Claim 20, drawn to the second method of use, radiotherapy.

The species have different structures and are expected to behave differently. Multiple products (protein and DNA molecule) and a second method of use of first product are claimed. Therefore, PCT/US95/11405 has a lack of unity pursuant of 37 CFR 1.475 (d).

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